# THE JOURNAL OF PHYSIOLOGY

EDITED FOR

## THE PHYSIOLOGICAL SOCIETY

BY


N. AMBACHE
R. V. COXON
M. DE B. DALY
P. M. DANIEL
S. P. DATTA
G. GORDON
G. W. HARRIS
R. C. JORDAN
B. KATZ
R. J. LINDEN
H. R. NOLTIE
W. J. O'CONNOR
W. D. M. PATON
W. A. H. RUSHTON
G. S. BRINDLEY (*Secretary*)
R. D. KEYNES (*Chairman*)



January 1961

Volume 155, No. 1

CAMBRIDGE UNIVERSITY PRESS

Bentley House, 200 Euston Road, London, N.W. 1

American Branch: 32 East 57th Street, New York 22, N.Y.

*Price 30s. net*

*(U.S.A. $5.00)*

PAGE

*Gaddum, J. H.* Push-pull cannulae . . . . . 1*P*

*Wright, B. M.* A vacuum bottle sampler for expired air . . . . . 2*P*

*Goad, A., Gregory, N. L. and McKay, J.* Transistorized drop-counter with simple drop chamber for measurement of blood flow . . . . . 3*P*

*Haselblatt, A. and Sproull, D. H.* Sedation and nystagmus produced by small doses of sodium barbital injected into the cerebral ventricles of rabbits . . . 3*P*

*Collins, A. R. J. and Stean, J. P. B.* A lever-operated, spring-loaded plunger clip for organ-bath drain pipes . . . . . 3*P*

*Bangham, D. R., Hobbs, K. R. and Tee, D. E. H.* The transmission of serum proteins from foetus to mother in the Rhesus monkey: indwelling cannulation of the foetus without interruption of pregnancy . . . . . 5*P*

*Ernsting, J., Gedye, J. L. and McHardy, G. J. R.* Further observations on brief profound anoxia . . . . . 3*P*

*Dornhorst, A. C.* Neurogenic vasodilation and willed movement . . . . . 5*P*

*Clitheroe, H. J. and Pickles, V. R.* The separation of the components of the menstrual stimulant . . . . . 5*P*

*Rushton, W. A. H.* Dark adaptation of human rods and the regeneration of rhodopsin 6*P*

*Hugh-Jones, P. and West, J. B.* Pulsatile gas flow in bronchi caused by the heart beat . . . . . 7*P*

*Andrews, T. M., Peart, W. S. and Robertson, J. I. S.* The release of serotonin by intravenous adrenaline and noradrenaline . . . . . 8*P*

*Axelsson, J.* The effect of nitrate on electrical and mechanical activity of smooth muscle . . . . . 9*P*

*Edman, K. A. P. and Schild, H. O.* Interactions of acetylcholine, adrenaline and magnesium with calcium in the contractions of depolarized rat uterus . . 10*P*

*Austen, K. F. and Brocklehurst, W. E.* Enhancement of the anaphylactic release of histamine and slow-reacting substance by certain dibasic fatty acids and inhibition by monobasic fatty acids . . . . . 12*P*

*Braun, C., Guzman, F., Horton, E. W., Lim, R. K. S. and Potter, G. D.* Visceral receptors, pain, bradykinin, and analgesic agents . . . . . 13*P*

*Hermansen, K.* The effect of adrenaline, noradrenaline and isoprenaline on the guinea-pig's uterus . . . . . 14*P*

*Telford, J. M. and West, G. B.* Studies with histidine decarboxylase . . . . 16*P*

*Barry, R. J. C., Dikstein, S., Matthews, J. and Smyth, D. H.* Electrical potentials in the isolated intestine . . . . . 17*P*

*Mills, J. N., Thomas, S. and Williamson, K. S.* Does spironolactone block the renal effects of cortisol? . . . . . 18*P*

*Bessou, P. and Laporte, Y.* Some observations on receptors of the soleus muscle innervated by Group III afferent fibres . . . . . 19*P*

*McConaghey, Paddy D. and Maizels, M.* Cation exchanges of human red cells . 20*P*

*Cooper, K. E., Cranston, W. I., Dempster, W. J. and Mottram, R. F.* Pyrogen-induced vasodilation in the transplanted kidney . . . . . 21*P*

*Mitchell, J. F.* Release of acetylcholine from the cerebral cortex and the cerebellum 22*P*

*Barrowman, J. and Roberts, K. B.* The foetal haemoglobins of mice . . . . 23*P*

*Wright, G. H.* Absorption and secretion of electrolytes in the stomach of the rabbit foetus . . . . . 24*P*

*Ashwood-Smith, M. J.* Viability of mouse bone marrow frozen to −79° C in the presence of dimethyl sulphoxide . . . . . 26*P*

**Notice to Contributors.** Papers submitted for publication should be sent to:

THE EDITORS OF THE JOURNAL OF PHYSIOLOGY

*Physiology Department, The Medical College of St Bartholomew's Hospital, Charterhouse Square, London, E.C.* 1

Contributors should follow the instructions in '**Suggestions to Authors**' with regard to the preparation of papers, and should study carefully the format of papers in recent numbers of the Journal. The latest edition of this pamphlet appeared in Vol. 150, p. 1. Offprints may be obtained from **The Cambridge University Press, 200 Euston Road, London, N.W. 1.** Price 2*s.* in paper cover, post free.

Papers submitted for publication should be accompanied by a declaration in the following terms: The attached paper entitled ..... has not been, and is not intended to be published anywhere except in the Journal of Physiology, and we/I agree that, if and when it is accepted by the Editors for publication, in consideration for such acceptance the entire copyright in this paper shall pass thereupon to the Physiological Society.

Requests for permission to reproduce any part of papers published in the Journal of Physiology should be addressed to the Secretary of the Editorial Board at the Physiological Laboratory, Cambridge. The Editorial Board will normally grant all requests from authors to reproduce material from their own papers. Permission will not be given to third parties except with the consent of the authors concerned, but if the Editorial Board receives no reply within a reasonable period of writing to the authors at their last address notified, consent will be assumed.

When a paper is submitted by several authors in collaboration their names will be printed in alphabetical order.

The date of receipt printed at the head of each paper is normally the date on which the typescript is first received by the Editors, but if a paper has to be extensively altered after submission the date may at the discretion of the Editors be changed to that on which an acceptable draft is received.

Authors are reminded that the acceptance of papers may be prevented by their being faulty in English composition. It is also essential that papers should be written as concisely as possible, without undue repetition of material in text, tables and figures. Details under the heading 'Methods' will appear in small print unless the author particularly wishes otherwise. Papers concerned with methods only are not acceptable, unless the methods involve new principles and are shown to be of proved value and interest to physiologists. Long theoretical discussions are in general unsuitable.

In an exceptional case the Editors will be prepared to accept a coloured illustration if they consider it to be necessary for conveying the scientific content of the paper. Half the cost of reproduction will be charged to the author.

Authors are requested to verify that the wording of references to unpublished work, and of acknowledgements of help or criticism, is approved by the persons concerned.

The Editors cannot accept responsibility for damage to or loss of papers submitted to them. Contributors are advised to keep copies of their typescripts and illustrations.

Proofs should be corrected and returned promptly. Unless otherwise directed, proofs are sent to the author whose name is first alphabetically, at the address from which the paper originates. If the author(s) wish the proofs to be sent to any other person, or to any other address, instructions should be given.

**Abstract.** Authors should send with their typescript an abstract suitable for inclusion in *International Abstracts of Biological Sciences*. This abstract will not appear in the *Journal of Physiology* but will be scrutinized by the Editors before being passed for publication in *International Abstracts*. It should contain a brief outline of the results and definitive conclusions of the work. Details of methods are generally not required. A paper of average length should be abstracted in about 100 words. The abstract should be typed in double spacing on a separate quarto sheet, and should be headed as follows: Title of paper; name(s) of author(s); *J. Physiol., Lond.*, followed by space for year, volume and page reference; abbreviated postal address including country of origin. For example:

A quantitative description of membrane current and its application to conduction and excitation in nerve. A. L. Hodgkin and A. F. Huxley. *J. Physiol., Lond.*, 1952, **117**, 500–544 (Physiol. Lab., Univ. Camb., Cambridge, England).—Text of abstract follows straight on.

The short title *J. Physiol., Lond.*, is used in the abstract to avoid confusion with *J. Physiol., Paris*, but should not be employed in the reference lists of papers submitted to the Journal.

PAGE

*Gaddum, J. H.* Push-pull cannulae . . . . . 1*P*

*Wright, B. M.* A vacuum bottle sampler for expired air . . . . . 2*P*

*Goad, A., Gregory, N. L. and McKay, J.* Transistorized drop-counter with simple drop chamber for measurement of blood flow . . . . . 3*P*

*Haselblatt, A. and Sproull, D. H.* Sedation and nystagmus produced by small doses of sodium barbital injected into the cerebral ventricles of rabbits . . . 3*P*

*Colline, A. R. J. and Stean, J. P. B.* A lever-operated, spring-loaded plunger clip for organ-bath drain pipes . . . . . 3*P*

*Bangham, D. R., Hobbs, K. R. and Tee, D. E. H.* The transmission of serum proteins from foetus to mother in the Rhesus monkey: indwelling cannulation of the foetus without interruption of pregnancy . . . . . 3*P*

*Ernsting, J., Gedye, J. L. and McHardy, G. J. R.* Further observations on brief profound anoxia . . . . . 3*P*

*Dornhorst, A. C.* Neurogenic vasodilation and willed movement . . . . . 5*P*

*Clitherow, H. J. and Pickles, V. R.* The separation of the components of the menstrual stimulant . . . . . 5*P*

*Rushton, W. A. H.* Dark adaptation of human rods and the regeneration of rhodopsin 6*P*

*Hugh-Jones, P. and West, J. B.* Pulsatile gas flow in bronchi caused by the heart beat . . . . . 7*P*

*Andrews, T. M., Peart, W. S. and Robertson, J. I. S.* The release of serotonin by intravenous adrenaline and noradrenaline . . . . . 8*P*

*Axelsson, J.* The effect of nitrate on electrical and mechanical activity of smooth muscle . . . . . 9*P*

*Edman, K. A. P. and Schild, H. O.* Interactions of acetylcholine, adrenaline and magnesium with calcium in the contractions of depolarized rat uterus . . 10*P*

*Austen, K. F. and Brocklehurst, W. E.* Enhancement of the anaphylactic release of histamine and slow-reacting substance by certain dibasic fatty acids and inhibition by monobasic fatty acids . . . . . 12*P*

*Braun, C., Gusman, F., Horton, E. W., Lim, R. K. S. and Potter, G. D.* Visceral receptors, pain, bradykinin, and analgesic agents . . . . . 13*P*

*Hermansen, K.* The effect of adrenaline, noradrenaline and isoprenaline on the guinea-pig's uterus . . . . . 14*P*

*Telford, J. M. and West, G. B.* Studies with histidine decarboxylase . . . . 16*P*

*Barry, R. J. C., Dikstein, S., Matthews, J. and Smyth, D. H.* Electrical potentials in the isolated intestine . . . . . 17*P*

*Mills, J. N., Thomas, S. and Williamson, K. S.* Does spironolactone block the renal effects of cortisol? . . . . . 18*P*

*Bessou, P. and Laporte, Y.* Some observations on receptors of the soleus muscle innervated by Group III afferent fibres . . . . . 19*P*

*McConaghey, Paddy D. and Maizels, M.* Cation exchanges of human red cells . 20*P*

*Cooper, K. E., Cranston, W. I., Dempster, W. J. and Mottram, R. F.* Pyrogen-induced vasodilation in the transplanted kidney . . . . . 21*P*

*Mitchell, J. F.* Release of acetylcholine from the cerebral cortex and the cerebellum 22*P*

*Barrowman, J. and Roberts, K. B.* The foetal haemoglobins of mice . . . 23*P*

*Wright, G. H.* Absorption and secretion of electrolytes in the stomach of the rabbit foetus . . . . . 24*P*

*Ashwood-Smith, M. J.* Viability of mouse bone marrow frozen to −79° C in the presence of dimethyl sulphoxide . . . . . 26*P*

**Notice to Contributors.** Papers submitted for publication should be sent to:

THE EDITORS OF THE JOURNAL OF PHYSIOLOGY

*Physiology Department, The Medical College of St Bartholomew's Hospital, Charterhouse Square, London, E.C.* 1

Contributors should follow the instructions in '**Suggestions to Authors**' with regard to the preparation of papers, and should study carefully the format of papers in recent numbers of the Journal. The latest edition of this pamphlet appeared in Vol. 150, p. 1. Offprints may be obtained from **The Cambridge University Press, 200 Euston Road, London, N.W. 1.** Price 2*s.* in paper cover, post free.

Papers submitted for publication should be accompanied by a declaration in the following terms: The attached paper entitled ..... has not been, and is not intended to be published anywhere except in the Journal of Physiology, and we/I agree that, if and when it is accepted by the Editors for publication, in consideration for such acceptance the entire copyright in this paper shall pass thereupon to the Physiological Society.

Requests for permission to reproduce any part of papers published in the Journal of Physiology should be addressed to the Secretary of the Editorial Board at the Physiological Laboratory, Cambridge. The Editorial Board will normally grant all requests from authors to reproduce material from their own papers. Permission will not be given to third parties except with the consent of the authors concerned, but if the Editorial Board receives no reply within a reasonable period of writing to the authors at their last address notified, consent will be assumed.

When a paper is submitted by several authors in collaboration their names will be printed in alphabetical order.

The date of receipt printed at the head of each paper is normally the date on which the typescript is first received by the Editors, but if a paper has to be extensively altered after submission the date may at the discretion of the Editors be changed to that on which an acceptable draft is received.

Authors are reminded that the acceptance of papers may be prevented by their being faulty in English composition. It is also essential that papers should be written as concisely as possible, without undue repetition of material in text, tables and figures. Details under the heading 'Methods' will appear in small print unless the author particularly wishes otherwise. Papers concerned with methods only are not acceptable, unless the methods involve new principles and are shown to be of proved value and interest to physiologists. Long theoretical discussions are in general unsuitable.

In an exceptional case the Editors will be prepared to accept a coloured illustration if they consider it to be necessary for conveying the scientific content of the paper. Half the cost of reproduction will be charged to the author.

Authors are requested to verify that the wording of references to unpublished work, and of acknowledgements of help or criticism, is approved by the persons concerned.

The Editors cannot accept responsibility for damage to or loss of papers submitted to them. Contributors are advised to keep copies of their typescripts and illustrations.

Proofs should be corrected and returned promptly. Unless otherwise directed, proofs are sent to the author whose name is first alphabetically, at the address from which the paper originates. If the author(s) wish the proofs to be sent to any other person, or to any other address, instructions should be given.

**Abstract.** Authors should send with their typescript an abstract suitable for inclusion in *International Abstracts of Biological Sciences*. This abstract will not appear in the *Journal of Physiology* but will be scrutinized by the Editors before being passed for publication in *International Abstracts*. It should contain a brief outline of the results and definitive conclusions of the work. Details of methods are generally not required. A paper of average length should be abstracted in about 100 words. The abstract should be typed in double spacing on a separate quarto sheet, and should be headed as follows: Title of paper; name(s) of author(s); *J. Physiol., Lond.*, followed by space for year, volume and page reference; abbreviated postal address including country of origin. For example:

A quantitative description of membrane current and its application to conduction and excitation in nerve. A. L. Hodgkin and A. F. Huxley. *J. Physiol., Lond.*, 1952, **117**, 500–544 (Physiol. Lab., Univ. Camb., Cambridge, England).—Text of abstract follows straight on.

The short title *J. Physiol., Lond.*, is used in the abstract to avoid confusion with *J. Physiol., Paris*, but should not be employed in the reference lists of papers submitted to the Journal.

# JOURNAL OF ENDOCRINOLOGY

## CONTENTS FOR PART 2 OF VOL. 21, NOVEMBER 1960. 27s. 6d.


ADAMS, C. W. M., FERNAND, V. S. V. and SCHNIEDEN, H. Changes in the adrenal and pituitary gland in experimental malnutrition.

EDWARDS, R. G. and FOWLER, RUTH E. Superovulation treatment of adult mice: their subsequent natural fertility and response to further treatment.

BRUSH, M. G. The effect of ACTH injections on plasma corticosteroid levels and milk yield in the cow.

BENTLEY, P. J. The effects of vasopressin on the short-circuit current across the wall of the isolated bladder of the toad, *Bufo marinus*.

SHUSTER, S. Antidiuretic hormone and corticotrophin release in man.

KORNER, A. The role of the adrenal gland in the control of amino acid incorporation into protein of isolated rat liver microsomes.

HARRISON, M. and FRASER, RUSSELL. Bone metabolism in rats, studied with stable strontium.

HARRISON, M. and FRASER, RUSSELL. Bone structure and metabolism in calcium-deficient rats.

HARRISON, M. and FRASER, RUSSELL. The parathyroid glands and calcium deficiency in the rat.

SHARP, G. W. G. The effect of light on diurnal leucocyte variations.

SHARP, G. W. G. The effect of light on the morning increase in urine flow.

RODECK, H., LEDERIS, K. and HELLER, H. The hypothalamo-neurohypophysial system in old rats.

KARAM, J., HARRISON, M. and FRASER, RUSSELL. The effect of reserpine on goitre induced by thiouracil.

GOOD, B. F., HETZEL, B. S. and OPIT, L. J. Effects of salicylate on plasma protein-bound iodine in thyroxine-maintained thyroidectomized rats.

DEANESLEY, RUTH. Endocrine activity of the early placenta of the guinea-pig.

PLUNKETT, E. R., SQUIRES, B. P. and RICHARDSON, S. J. The effect of relaxin on thyroid weights in laboratory animals.


---

## CONTENTS FOR PART 3 OF VOL. 21, DECEMBER 1960. 27s. 6d.


KUSHINSKY, S. and WU, JANE. Effect of alkali on urinary oestrogens.

PHILLIPS, R. E. and TIENHOVEN, A. VAN. Endocrine factors involved in the failure of pintail ducks *Anas acuta* to reproduce in captivity.

BROOKS, R. V. and PRUNTY, F. T. G. Patterns of steroid excretion in three types of post-pubertal hirsutism.

BROOKS, R. V. The metabolism of 17α-hydroxyprogesterone in congenital adrenal hyperplasia.

PAUL, J. and PEARSON, ENID S. The action of insulin on the metabolism of cell cultures.

WOODHEAD, A. D. The presence of adrenocorticotrophic hormone in the pituitary of the Arctic cod, *Gadus morhua* L.

GORE, MARION B. R. and BARON, D. N. Estimation and identification of free and conjugated corticosteroids in human plasma.

EDGAR, D. G. and ASDELL, S. A. The valve-like action of the utero-tubal junction of the ewe.

EDGAR, D. G. and ASDELL, S. A. Spermatozoa in the female genital tract.

BRAUNSBERG, HANNELORE and JAMES, V. H. T. The determination of adrenocortical steroids in blood. Results in normal individuals and adrenal hyperfunction.

LINDNER, H. R. and MANN, T. Relationship between the content of androgenic steroids in the testes and the secretory activity of the seminal vesicles in the bull.

MANN, T., ROWSON, L. E. A. and HAY, MARY F. Evaluation of androgenic and gonadotrophic activity in male twin calves by analysis of seminal vesicles and semen.

*Proceedings of the Society for Endocrinology.*



*Subscriptions (90s. per volume of 4 parts) should be sent to the*

CAMBRIDGE UNIVERSITY PRESS

BENTLEY HOUSE, 200 EUSTON ROAD, LONDON, N.W.1

**BLOOD FLOW IN ARTERIES**

D. A. McDONALD

*Reader in Physiology, St Bartholomew's Hospital, University of London*

— the Seventh of the Monographs of the Physiological Society.

Contents include the nature of flow in a liquid, the characteristics of blood flow in the circulation, the disturbances in blood flow due to the configuration of the vascular bed, the pressure-flow relationship of oscillatory flow, the measurement of phasic flow velocity, the pulsatile flow pattern in arteries and the physical properties of the arterial wall.

**320 pages 70 illustrations**

**40s. net**

**EDWARD ARNOLD**

*Particulars of the*

*JOURNALS*

*Published by the Cambridge University Press, may be had from*

THE MANAGER
CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE
200 EUSTON ROAD
LONDON, N.W.1

THE BRITISH JOURNAL OF NUTRITION

**PUBLISHED FOR THE NUTRITION SOCIETY**

*Vol. 15, No. 1 Shortened Versions of Titles 1961 35s. net*

Metabolic studies on large and small eaters. By GEOFFREY A. ROSE and R. T. WILLIAMS.

Protein concentration and protein value of diets. By D. S. MILLER and P. R. PAYNE.

Basal metabolism in obesity and anorexia nervosa. By H. LJUNGOREN, D. IKKOS and R. LUFT.

Nutrition of the cat. 4. By PATRICIA P. SCOTT, J. P. GREAVES and M. G. SCOTT.

Weight reduction on different diets. By R. F. FLETCHER, MARJORIE Y. MCCRIRICK and A. C. CROOKE.

Copper metabolism in young pigs given copper sulphate or sulphide. By J. P. BOWLAND, R. BRAUDE, A. G. CHAMBERLAIN, R. F. GLASCOCK and K. G. MITCHELL.

Nutrition of the cat. 5. By A. HEULWEN ROBERTS and PATRICIA P. SCOTT.

Nitrogen and mineral retentions by babies. By JEAN E. SLATER.

Treatment of ewes during pregnancy with cobaltic-oxide pellets, composition of milk and growth of lambs. By M. W. O'HALLORAN and K. D. SKERMAN.

Effect of plane of energy nutrition of the cow on constituents of milk and blood plasma. By J. A. F. ROOK and C. LINE.

Severe undernutrition in growing and adult animals. 6. By C. W. M. PRATT and R. A. MCCANCE.

Vitamin $D_3$ and absorption of calcium in the chick. By M. E. COATES and E. S. HOLDSWORTH.

Rachitogenic factor and calcium metabolism in chicks. By M. E. COATES, G. F. HARRISON and E. S. HOLDSWORTH.

*Combined Subscription for The British Journal of Nutrition (4 issues) and The Proceedings of the Nutrition Society (2 issues) is £7 per annum.*

**Cambridge University Press, Bentley House, 200 Euston Road, London, N.W.1**

# THE JOURNAL OF PHYSIOLOGY

★ The subscription price, for orders placed in advance of publication, for vols. 155, 156, 157, 158, 159 (1961) is 80*s*. per volume of 3 parts post free ($13.50 in the U.S.A.).

In normal circumstances, five volumes will appear in each year.

Orders may be placed with your Bookseller, or sent direct to the
**Cambridge University Press,**
**Bentley House, 200 Euston Road, London, N.W. 1**

★ Also available from the **Cambridge University Press**
Back numbers from vol. 61 (1926) onwards.
Volumes 61–115, price £4. 10*s*. per volume.
Volumes 116–120, price £4 per volume.
Volumes 121–130, price £3. 15*s*. per volume.
Volumes 131–154, price £4. 10*s*. per volume.
Single issues and complete sets can be supplied.

Author and subject indexes to the Journal of Physiology, prepared by Dr Grace Eggleton.
Vols. 61–100. 30*s*. net.
Vols. 101–115. 30*s*. net.

★ Available from **Wm. Dawson & Sons, Ltd.**
**16 West Street, Farnham, Surrey, England**

Back numbers, vols. 1–25. Price £6 per volume.
Members' rates on application.

Ready shortly, vols. 20–60 bound, £8 per volume.

Some back issues and volumes are available from Volumes 1–60, particulars on request.

Author index of the Journal of Physiology, vols. 1–60. 25*s*. net.

Subject index of the Journal of Physiology, vols. 1–60, prepared by Dr J. G. Priestley. 15*s*. net.

History of the Physiological Society during its first fifty years 1876–1926. By Sir Edward Sharpey-Schafer, F.R.S. 15*s*. net.

*J. Physiol.* (1961), **155**, *pp.* 1–27
*With* 11 *text-figures*
*Printed in Great Britain*



# THE OUTPUT OF SYMPATHETIC AMINES FROM THE CAT'S ADRENAL GLAND IN RESPONSE TO SPLANCHNIC NERVE ACTIVITY

BY E. MARLEY AND W. D. M. PATON

*From the Department of Pharmacology, Royal College of Surgeons of England, Queen Square, London, W.C.* 1

(*Received* 25 *April* 1960)

The release of sympathins from the adrenal gland as a consequence of electrical stimulation of the splanchnic nerve was first tested by Biedl (1897), Dreyer (1899) and Tscheboksaroff (1911). These were followed by the fuller investigations of Elliott (1912, 1913). Amongst other observations he found that, after prolonged excitation of the splanchnic nerve, the effectiveness of excitation was somewhat reduced, but he could not demonstrate any depletion of the adrenaline content of the adrenal gland. After these pioneer studies little attention seems to have been paid to the release of sympathetic amines by splanchnic nerve stimulation until recently. This cannot entirely be ascribed to lack of sensitive assay techniques, for Stewart & Rogoff (1917), investigating the resting output of the gland, had used the rabbit intestine and uterus as test objects for assay, and the great sensitivity to adrenaline of the fowl's isolated rectal caecum was exploited by Barsoum & Gaddum (1935). The realization that noradrenaline as well as adrenaline is released from the adrenal gland (Bülbring & Burn, 1949), together with the introduction of more sensitive assay techniques such as those using the rat uterus (de Jalon, Bayo & de Jalon, 1945) and the pithed rat blood pressure (Shipley & Tilden, 1947) have allowed a more precise assessment not only of the resting output of the gland (Vogt, 1952) but also of its output during electrical excitation (Outschoorn, 1952; Rapela, 1956; Eade & Wood, 1958). Investigation of the gland's output by pharmacological assay of the substances released into the adrenal blood can be made as rigorous as desired, but it is laborious and does not allow the time relationship of release to be easily determined. The method recently developed by Vane (1958), by which a strip of tissue sensitive to the amines can be included in a small extracorporeal circulation, offered an additional and more convenient method of estimating amine output. We have therefore used both methods in experiments to investigate the relation between output of adrenal medullary hormones

and various patterns of electrical stimulation, and also the susceptibility of the ganglionic synapse to fatigue and to specific blocking agents.

## METHODS

Cats were anaesthetized with ether, after induction by ethyl chloride, and then given chloralose intravenously (80 mg/kg). A few animals were anaesthetized with intraperitoneal pentobarbitone sodium (30 mg/kg). For the collection of samples of blood from the adrenal vein, the abdomen was opened down the mid line, the stomach, intestines, spleen and pancreas were removed, and the blood supply through the hepatic artery was arrested. The vessels around the left adrenal gland were dissected out and branches joining the adrenolumbar vein lateral to the gland, either from the diaphragm, the abdominal wall or the renal vein, were tied off. Artificial ventilation was begun. By dissection under the diaphragm the left greater splanchnic nerve was freed and a loose ligature left round it as high as possible. The lesser splanchnic nerves and the sympathetic chain on the same side were also cut. The animal was then given heparin intravenously (10 mg/kg). A siliconed polythene cannula was inserted into the adrenolumbar vein just lateral to the adrenal gland; when the adrenal vein was then tied close to the inferior vena cava, blood from the gland flowed into the cannula. Because of the amount of residual blood in the cannula at the end of a control or stimulation period, which would otherwise be included with the next specimen, the cannula was provided with a side arm close to its insertion into the vein, whereby at the conclusion of the collection period the contained blood could be gently blown out. After the collection of (usually) two control specimens the splanchnic nerve was cut and the peripheral end laid across platinum electrodes (cathode distal) for stimulation with supramaximal rectangular (0·5 msec) pulses of 20 V, at varying frequencies. In experiments where a brief burst of rapid excitation was required two Bell square-wave stimulators were linked, the one providing the required stimulation frequency and the other a variable 'gate', up to 500 msec duration during which a relay was closed connecting the first stimulator to the electrodes. Stimuli were monitored with a Cossor oscilloscope to verify the absence of wave distortion.

Blood pressure was recorded throughout the experiment with a siliconed cannula in the right carotid artery as a check against any leak of pressor amines from the prepared gland into the general circulation.

Samples of venous effluent were collected in chilled siliconed graduated centrifuge tubes containing 1 mg heparin and standing in ice. They were immediately centrifuged at 3000 rev/min for 10 min in containers with ice (Gaddum, Peart & Vogt, 1949), to prevent the development of vasopressor activity unrelated to sympathin. After centrifugation plasma and cell volumes were noted and the plasma was pipetted off and kept in sealed bottles on ice until assay.

Circulating sympathins were assayed directly by the blood-bathed isolated organ technique of Vane (1958). The left greater splanchnic nerve was approached through a lumbar incision, after ligature and section of the posterior abdominal muscles. The adrenolumbar vein was divided between ligatures, and the greater splanchnic nerve identified and cut, together with the lesser splanchnic nerves and lumbar sympathetic trunk. The abdomen was then opened in the mid line and the right adrenal gland removed. After the injection of heparin (10 mg/kg) the left carotid artery was cannulated (the right carotid artery being employed for recording blood pressure) and connected with polythene tubing through a roller pump calibrated to deliver 10–15 ml. of blood/min to a siliconed organ bath, maintained at 37·5° C by an external water jacket and containing a rat-stomach strip preparation (Vane, 1957); this is approximately twice as sensitive to adrenaline as it is to noradrenaline. The blood overflowed from the organ bath through a side arm and dripped over the surface of another isolated organ, usually the chick rectum (West, 1950) which is 60–100 times more sensitive to adrenaline than it is to noradrenaline, before being returned by gravity feed to the right

external jugular vein. In the early experiments with blood-bathed tissues the extracorporeal circulation contained 10–15 ml. of blood; later this was modified, reducing the amount of blood circulating outside the body to 5–10 ml. The tone of the rat stomach strip and the chick rectum, which both contract when bathed with blood, was recorded by an auxotonic pendulum lever (Paton, 1957) of 16:1 magnification. A vibrator was mounted on the stand holding the two levers, to minimize friction between the writing points and the kymograph. For prolonged splanchnic stimulation the current to and from the electrodes was passed through 1 $\mu$F condensers, to prevent polarization of the electrodes (Garry & Gillespie, 1955). In all experiments the animals were artificially ventilated and their temperature was maintained at 38° C.

Noradrenaline was estimated by its pressor effect on pithed rats (Shipley & Tilden, 1947). Rats of 200–250 g were anaesthetized with ether and injected subcutaneously with 1 mg hyoscine. A tracheal cannula was inserted for artificial ventilation and the central nervous system was destroyed by introducing a wire rod into one orbit and through the foramen magnum into the spinal cord. Such rats have a blood pressure of 50–60 mm Hg. A venous cannula was placed in the left jugular or right femoral vein, and the arterial blood pressure was recorded from the right carotid artery. The venous cannula was connected through rubber tubing to a 5·0 ml. microburette filled with NaCl solution 0·9 g/100 ml. so that drugs or plasma samples injected through the rubber tubing could be washed into the animal. The total volume injected was always adjusted to 0·4 ml.

To assay adrenaline the rat uterus, in a 10 ml. bath, stimulated with acetylcholine or carbachol was originally employed (de Jalon *et al.* 1945); this is about 150 times more sensitive to adrenaline than to noradrenaline (Gaddum *et al.* 1949). It is now known (Vane, 1957) that there is in cat plasma about 2 $\mu$g/ml. of 5-hydroxytryptamine (5-HT); since this substance causes the rat uterus to contract, in later experiments bromlysergic acid $10^{-9}$ was left in the bath for 15–30 min, until (2·5 $\mu$g) 5-HT became ineffective.

The largest volume of plasma used was 0·3 ml. containing $< 0{\cdot}6$ $\mu$g 5-HT (Expts. 11, 13, 14). In other experiments (Nos. 15, 16) adrenaline was assayed by its reduction of maximum contractions of the rat uterus evoked by 5-HT, thus nullifying the effect of 5-HT in the plasma samples. The use of a pendulum auxotonic lever (Paton, 1957) assisted in regularizing contraction heights.

For both adrenaline and noradrenaline the assay was always done by bracketing the unknown solutions between doses of the standard differing as a rule by a factor of two, and assaying at two dose levels. Random specimens showing adrenaline-like activity were treated with Lugol's iodine and allowed to stand 5 min at room temperature. Excess iodine was destroyed with thiosulphate and the samples were retested to demonstrate the disappearance of adrenaline-like activity. The results are expressed as the amount of adrenaline and noradrenaline in the plasma that flowed from the suprarenal gland in 1 min.

In dealing with amine mixtures the content of each amine is obtained from a knowledge of the noradrenaline and adrenaline equivalents on two discriminating test objects. From the equivalents two simultaneous equations may be set up and solved for the individual amine contents. A simpler graphical method is to make on linear graph paper a scale of noradrenaline equivalent per millilitre on one axis, and of adrenaline equivalent per millilitre on the other axis. For a given sample on a given test object a line can then be drawn joining the two equivalents; this line will pass through all the points corresponding to various noradrenaline–adrenaline mixtures which would have the same effect. A corresponding line is drawn for the same sample on the other test object and the point of intersection gives the independent amine contents. The method is quick and the slopes of the lines make obvious the different discriminating power of the two test objects. *Drugs* used were adrenaline bitartrate, noradrenaline bitartrate, acetylcholine chloride, bromlysergic acid (BOL 148), carbachol chloride, histamine acid phosphate, hyoscine hydrobromide, methonium salts ($C_4$, $C_5$, $C_7$, $C_{10}$) as iodides except for hexamethonium bromide, nicotine

hydrogen tartrate, probanthine bromide, serotonin creatinine sulphate, tetraethylammonium bromide (TEA), D-tubocurarine chloride. Doses are given as the amount of salt, except for adrenaline, noradrenaline, histamine and serotonin, which are measured as base.

## RESULTS

### *Resting output from the adrenal gland before and after splanchnic nerve section*

Before testing the responses of the glands to splanchnic nerve stimulation the opportunity was taken in 13 experiments of estimating the resting output of sympathins from the gland before nerve section. At least two samples were taken before the nerve was divided: Table 1 gives the figures obtained. There appeared to be a tendency for the output in the last control sample to be higher than the preceding ones; we believe, however, that the last control samples give the best values, since they were taken as long as possible (20–88 min) after completing the dissection, which might depress output for some time. In chloralosed cats output ranged from 3 to 198 ng noradrenaline/min and from < 1 to 209 ng adrenaline/min; with phenobarbitone sodium (cats 7, 8) the corresponding figures were noradrenaline 2–141 ng/min and adrenaline 1–99 ng/min. In the period 9–15 min after nerve section the output almost always fell unless it was initially barely detectable. On the other hand, output rarely became undetectable; and in eight experiments the output subsequently rose again in the absence of nerve excitation. For the whole group only the fall in noradrenaline output immediately following nerve section was significant ($t = 2{\cdot}26$, $P < 0{\cdot}05$).

The problem arose as to the reason for sustained output, sometimes considerable, in an acutely denervated gland. Asphyxia, circulating histamine or a rise in plasma potassium could all be excluded. Changes in blood flow through the gland may influence output. But we found no relation between changes in output and blood-flow rate: thus in one experiment the blood flow rose from 0·3 to 0·48 ml./min, and the total amine output rose from 45 to 101 ng/min; in another experiment blood flow fell from 0·21 to 0·12 ml./min, and the total amine output rose from 6 to 42 ng/min. All these figures refer to periods after dividing the nerve and before any stimulation. It may be that the rate of plasma flow rather than whole blood flow is more intimately linked with amine output. The mean plasma flow for the specimens before nerve section was 0·23 ml./min (25 samples) and after nerve section but preceding stimulation it was 0·215 ml./min (26 samples). Since there was no significant change in mean plasma flow, it is unlikely that the above changes in amine output depended on plasma-flow fluctuations.

Three possibilities remain. First, the denervation could have been

TABLE 1. Resting output of noradrenaline and adrenaline (ng/min) before and after splanchnic nerve section

| | Expt. No. ... | 1 | | 2 | | 4 | | 5 | | 6 | | 7 | | 8 | | 10 | | 11 | | 13 | | 14 | | 15 | | 16 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sample | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Before nerve section | 1 | 70 | 31 | 49 | 30 | 19 | <1 | 8 | 3 | 57 | 84 | 39 | 40 | 4 | 4 | 11 | 3 | 17 | 20 | 3 | <2 | 38 | 17 | 134 | 86 | — | — |
| | 2 | 198 | 160 | 50 | 29 | 23 | <1 | 5 | 3 | 190 | 209 | 141 | 99 | 2 | 1 | 14 | 7 | 4 | 15 | 3 | 1 | 28 | 2 | 101 | 50 | 83 | 7 |
| After nerve section | 1 | 26 | 9 | 6 | 39 | 14 | <1 | 3 | 2 | 32 | 115 | 29 | 32 | 28 | 1 | 1 | 1 | 4 | 2 | 3 | 1 | 5 | <1 | 67 | 24 | 21 | 10 |
| | 2 | 14 | 7 | 52 | 39 | 20 | 12 | 4 | 3 | 83 | 98 | 131 | 498 | 4 | 4 | 1 | 4 | 9 | 7 | 4 | 4 | 44 | 17 | 11 | 14 | 7 | 11 |

| | Sample | Mean values (ng/min) NAd | Ad |
|---|---|---|---|
| Before section | 1 | 37·4 | 26·7 |
| | 2 | 64·8 | 44·9 |
| After section | 1 | 18·4 | 17·6 |
| | 2 | 29·5 | 56·2 |

incomplete; but all homolateral nerves known to influence secretion had been cut, and there is no evidence for contralateral innervation of the gland (Young, 1939). Secondly, since chloralose depresses the secretion by the gland (Malmejac & Neverre, 1950) it might be that its elimination in the body allowed a non-nervous element in the secretion to rise, but the action of chloralose is so prolonged that this seems unlikely. Thirdly, it is possible that the gland was in a state of mild 'exhaustion' before nerve section, sufficient to reduce *spontaneous* secretion, and that after a rest period following the section, spontaneous secretion at a higher rate could be resumed. This was borne out by the observation that the rise in resting secretion after nerve section tended to be high in those animals which had high outputs before section. This idea would imply that the non-nervous secretion represents a sort of overspill of amines from a gland, as soon as its stores are full.

*The threshold frequency of excitation and number of shocks for the release of significant quantities of sympathetic amines*

Although excitation of the splanchnic nerve at 10/sec produces a vigorous release of both adrenaline and noradrenaline, the output of these hormones with excitation at 1/sec usually does not increase output above the resting unstimulated level. An attempt was made, therefore, to determine the frequency of stimulation at which a consistently increased output could be obtained. Table 2 gives the results, which can be summarized as follows. Excitation at 1/sec for 5–15 min (totals of 300–900 shocks) produced a significantly increased output in three out of six experiments, whereas at 2/sec output was increased in all of three tests, and at 4/sec in all of eight tests. When the number of shocks was reduced to a total of 60 (excitation at 1/sec for 1 min), output did not increase in three tests. It appears, therefore, that only with prolonged excitation will a frequency of 1/sec occasionally prove effective, and that 2/sec is probably the lowest frequency which consistently increases the output from the medulla.

This conclusion was further tested in experiments with blood-bathed assay tissues (rat stomach strip, chick rectum) in an extra-corporeal circulation, and similar results were obtained. Excitation at 1/sec for 5 min was effective in one test; but in six tests with excitation for times varying from 30 sec to 3 min, no significant release of amines occurred. With a frequency of 2/sec, applying 60–180 shocks, release occurred in three out of four experiments; and at 3/sec 60 shocks were invariably effective (seven experiments).

To determine the minimum number of shocks which can cause a detetectable output, it is necessary to know the optimum frequency of stimulation. This was found (as described below) to be about 30–60/sec.

TABLE 2. Total amine output (ng/min) in adrenal venous blood in control and stimulation specimens after splanchnic nerve section. In parentheses, duration (min) of collection period*. Lower figures represent values in control period succeeding stimulation except in Expt. 4 where lower figure is for a later stimulation period at 1/sec

| Expt. No. | Control total amine output | Total amine output on splanchnic nerve stimulation | | | | | | | Duration of stimulation or total stimuli |
|---|---|---|---|---|---|---|---|---|---|
| | | 1/sec | 2/sec | 4/sec | 8/sec | 10/sec | 16/sec | 32/sec | |
| 1 | 28 | 29 (20) | — | — | — | 717 (15) | — | — | 15 min |
| 2 | 95 | 120 (15) | — | — | — | 1452 (20) | — | — | 10 min |
| 4 | 23 | 148 (22) | 158 (15) | 288 (14) | 702 (12) | — | 86 (13) | — | 10 min at 2, 4, 8, 16/sec |
| | | 174 (12) | | | | | | | 15 min at 1/sec |
| 5 | 7 | 44 (31) | 81 (21) | 92 | 157 (12) | — | 89 | 43 (12) | 8 min |
| 6 | 157 | 181 (11) | — | — | — | — | — | — | 10 min |
| 7 | 345 | — | — | 938 (19) | 1804 (14) | 3248 | 3139 (11) | — | 10 min |
| | | | | 1614 (14) | 1182 (11) | 568 (12) | | | |
| 8 | 6 | 98 (11) | 70 (14) | — | — | — | — | — | 5 min |
| | | 31 (14) | 47 (12) | | | | | | |
| 10 | 4 | 6 | — | 20 | — | — | 6 | 9 | 60 stimuli |
| | | | | 8 | | | 5 | 12 | |
| 11 | 39 | 141 | — | 245 | — | — | 270 | 204 | 180 stimuli |
| | | | | 82 | | | 74 | 147 | |
| 13 | 6 | 6 | — | 20 | — | — | 40 | 100 | 180 stimuli |
| | | | | | | | | 13 | |
| 14 | 28 | 17 | — | 24 | — | — | 80 | 71 | 60 stimuli |
| | | | | | | | 34 | 65 | |
| 15 | 51 | 40 | — | 67 | — | — | 48 | 215 | 60 stimuli |
| | | | | | | | 147 | 116 | |

* Collection period was 10 min unless otherwise stated.

With this range of rates, 60 stimuli always produce a detectable output, sometimes large. With fewer stimuli sometimes no response occurs; but in one experiment 13 shocks at 128/sec caused a detectable response (Fig. 1).

*Relation of output to varying rates and durations of stimulation*

*Number of stimuli kept constant; experiments with adrenal vein cannulation.* Five experiments were performed, three (Nos. 10, 14, 15) in which a total of 60 stimuli were given and two (Nos. 11, 13) with 180 stimuli, at stimulation rates of 1, 4, 16 and 32/sec, in random order to avoid systematic error,

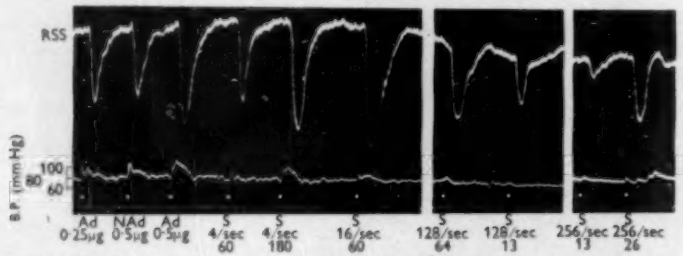



Fig. 1. Cat, 2·6 kg; chloralose. Response of blood pressure (B.P.) and of the blood-bathed isolated rat stomach strip (RSS) to intravenous injections of adrenaline or noradrenaline or to stimulations of the cut left splanchnic nerve at various frequencies. Total number of shocks 13, 26, 60, 64 or 180, as shown. Time marker, minutes.

due for instance, to the deterioration of the animal during the experiment. Both stimulation and control specimens were collected for 10 min periods. An arbitrary control value was arrived at by averaging the results for the two control specimens preceding the first stimulation and that for the control period succeeding excitation at 1/sec. Difficulty arises in a statistical analysis of the results because with the higher frequencies of stimulation considerably increased outputs of amines are to be found in the immediately succeeding control specimens. This could not be attributed to blood belonging to the stimulation specimen remaining in the dead space of the cannula in the adrenal vein, with subsequent carry-over into the next control sample. A correction was applied by adding to the stimulation output the excess found in the first control period after stimulation over the averaged control value. The mean values for the corrected total amine output (ng/min) in the control and stimulation specimens, and their range, are shown in Table 3. Analysis of variance of the data shows that the results for the control and stimulated specimens depart from homogeneity ($P < 0{\cdot}01$); and, as might be expected, there is a significant contribution to the total variance from the difference between the experimental animals ($P < 0{\cdot}001$). Comparison of the mean values (using a one-tailed $t$ test)

shows that there is no significant difference between the total amine output in the control and stimulation at 1/sec specimens ($t = 0{\cdot}62$, $P = > 0{\cdot}5$), but that the differences between the total amine output in the control and stimulation specimens at 4/sec ($t = 1{\cdot}78$, $P < 0{\cdot}05$), 16/sec ($t = 2{\cdot}75$, $P < 0{\cdot}001$) and 32/sec ($t = 4{\cdot}19$, $P < 0{\cdot}001$) are significant, the significance mounting with frequency of stimulation. Analysis of variance of only the stimulation specimens showed them to depart significantly from homo-

TABLE 3. Combined adrenaline and noradrenaline outputs* (means and range, ng/min to the nearest whole figure) for the control and stimulation specimens after splanchnic nerve section. (Combined results of Expts. 10, 14, 15 (60 shocks) and Expts. 11, 13 (180 shocks).)

| Indices | Control ($n = 15$) | 1/sec ($n = 5$) | 4/sec ($n = 5$) | 16/sec ($n = 5$) | 32/sec ($n = 5$) |
|---|---|---|---|---|---|
| Mean | 26 | 46 | 85 | 117 | 165 |
| Range | 2–91 | 6–162 | 20–288 | 7–305 | 17–312 |

* Corrected as explained in text.

TABLE 4. Comparison of the mean combined adrenaline and noradrenaline outputs (ng/min; corrected) for the control and stimulation specimens with either 60 or 180 shocks after splanchnic nerve section

| Total stimuli | Control | 1/sec | 4/sec | 16/sec | 32/sec |
|---|---|---|---|---|---|
| 60 shocks (Expts. 10, 14, 15) | 28 | 21 | 38 | 79 | 135 |
| 180 shocks (Expts. 11, 13) | 23 | 84 | 154 | 174 | 210 |

geneity ($F = 7{\cdot}00$, $P < 0{\cdot}01$). Comparison of the mean values for the stimulation specimens (using a two-tailed $t$ test) indicated that, whereas output of the 16/sec or 32/sec specimens differed significantly from that of the 1/sec specimen, and that of the 32/sec from the 4/sec specimen, the differences between the mean output at 1/sec and 4/sec, 4/sec and 16/sec and 16/sec and 32/sec were not significant. The data from these experiments were further subdivided to compare the mean total amine outputs for stimulation with 60 shocks with those occurring with 180 shocks to the cut splanchnic nerve (Table 4). As expected, outputs were found to be higher with the larger number of stimuli. Figure 2 shows the mean outputs at varying frequencies, plotted, not against frequency, but against the interval between successive shocks, since it will be discussed in these terms.

*Proportionate outputs of adrenaline and noradrenaline.* So far we have considered total amine output; the uncombined adrenaline and noradrenaline outputs (corrected) in the experiments just discussed were also calculated separately. The means, ranges and standard errors of the mean for these outputs, together with values for the ratio between the two amines, are shown in Table 5. Output of both amines rose with increasing frequency of stimulation, and there was again a considerable variation between different animals. Although a fall in the ratio of mean nor-

adrenaline to mean adrenaline output occurred at 32/sec stimulation, this was far from significant statistically, and our main conclusion is that the ratio does not change with brief periods of excitation at the serates. Since, however, Rapela (1956) has reported a specific increase in adrenaline at

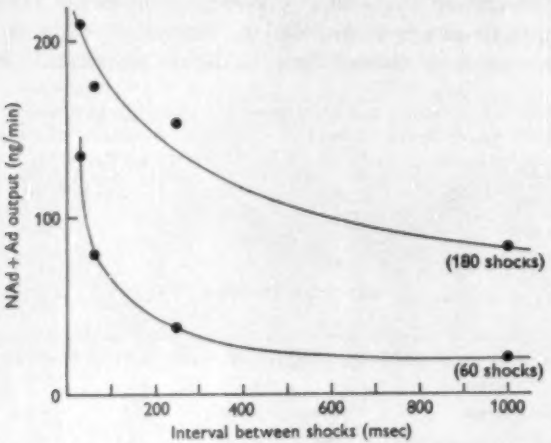



Fig. 2. The relation between the mean output of combined adrenaline and noradrenaline in the suprarenal venous blood (ng/min) to the interval (msec) between shocks administered to the cut left splanchnic nerve.

TABLE 5. The ratio of the means, the mean, the range and standard errors of the mean for the separate corrected noradrenaline and adrenaline outputs (ng/min) in the control and stimulation specimens after splanchnic nerve section (Expts. 10, 11, 13, 14, 15)

| Indices | Control ($n = 15$) | 1/sec ($n = 5$) | 4/sec ($n = 5$) | 16/sec ($n = 5$) | 32/sec ($n = 5$) |
|---|---|---|---|---|---|
| Noradrenaline | | | | | |
| Mean | 15·1 | 30·6 | 43·0 | 65·2 | 70·0 |
| Range | 1–67 | 3–117 | 4–152 | < 1–211 | 1–180 |
| S.E. of mean | 4·87 | 19·49 | 24·75 | 33·89 | 30·98 |
| Adrenaline | | | | | |
| Mean | 10·5 | 16·6 | 42·4 | 52·0 | 96·4 |
| Range | < 1–27 | 3–44 | 7–135 | 3–95 | 17–208 |
| S.E. of mean | 2·39 | 7·08 | 21·12 | 20·75 | 31·76 |
| Ratio noradrenaline mean: adrenaline mean | 1·4 | 1·8 | 1·0 | 1·2 | 0·7 |
| Range of ratio, noradrenaline: adrenaline | 0·05–5·0 | 0·75–2·7 | 0·19–2·0 | 0·14–13·3 | 0·06–3·5 |

high rates of excitation, this point was examined further; and in one experiment in which 51 shocks at a rate of 512/sec were given, an increase in output of adrenaline alone from 11 to 59 ng/min was obtained and in a second test from 17 to 44 ng/min; the noradrenaline output remained constant at about 18 ng/min. At 256/sec 26 shocks had a doubtful effect of the same

kind; 13 shocks at 128/sec were ineffective. It seems likely, therefore, that a selective increase in adrenaline secretion can be obtained, but not at frequencies of stimulation of physiological interest.

*Effect on output of the plasma volume of the venous sample.* Brown & Gillespie (1957) noted that even when both number of stimuli and frequency of excitation were kept constant, the noradrenaline output varied directly with the plasma volume of the sample but not with the whole-blood volume of the sample. This variation of output with sample volume might introduce serious errors in the interpretation of the results if, for instance, the sample volumes at the higher frequencies of stimulation were

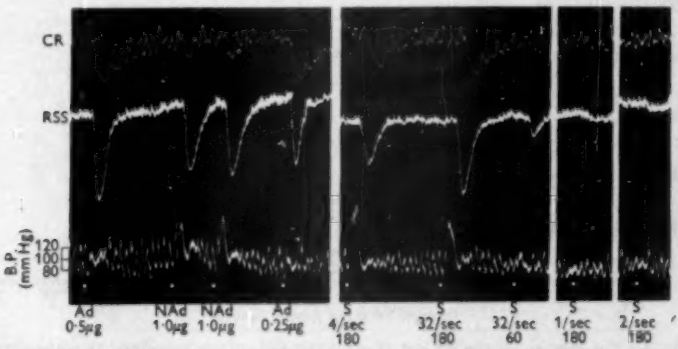



**Fig. 3.** Cat, 2·5 kg; chloralose. The response of the blood-bathed isolated chick rectum (CR), rat stomach strip (RSS) and blood pressure (B.P.) to intravenous injections of adrenaline or noradrenaline, or to stimulation of the cut left splanchnic nerve at various frequencies, for either 60 or 180 shocks. Time marker, minutes.

consistently greater than those of the lower. In fact, however, there was no consistent change of flow rate; the average plasma flow rate for the control period and stimulation at 1, 4, 16, and 32/sec respectively were 0·11, 0·11, 0·08, 0·08 ml./min in the experiments analysed above. There was a small reduction of flow with excitation at 16 or 32/sec, and so the values for output at these rates may be slightly underestimated.

*Experiments with blood-bathed assay tissues*

The observations just described, showing that excitation with a fixed number of shocks led to a higher output of amines if the rate of stimulation was increased, were extended by the less laborious assay technique of the blood-bathed organ. The comparative effect of stimulation at varying frequencies with either 60 or 180 shocks can be seen from Fig. 3, and Fig. 4 shows outputs determined by this method in three experiments. The same general result was obtained, that output is highest for frequencies of 30–60/sec (Fig. 5). A further experiment was made (Fig. 6) in which 50, 150, or

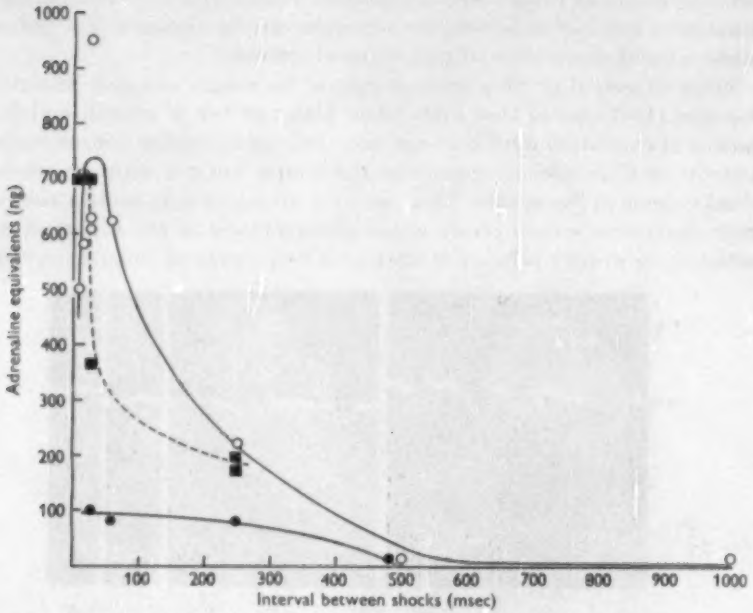



*Fig. 4.* The relation of the output of sympathetic amines (measured as adrenaline equivalents on the blood-bathed isolated rat stomach strip) to the interval between shocks given to the cut left splanchnic nerve. ●—● = 60 shocks; ■—■ and ○—○ = 180 shocks.

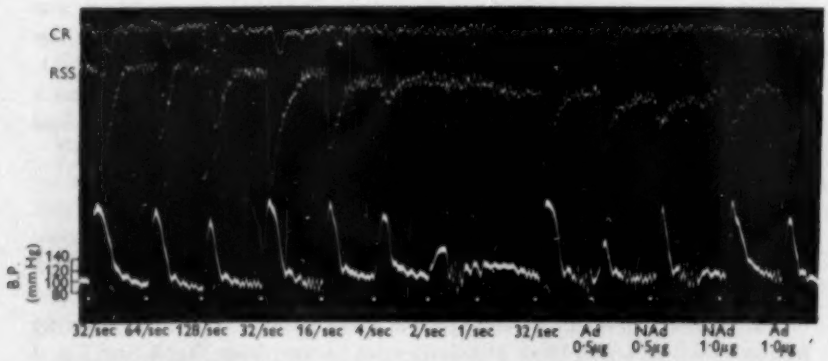



*Fig. 5.* Cat, 2·3 kg; chloralose. The response of the blood pressure (B.P.) and of the blood-bathed isolated chick rectum (CR) and rat stomach strip (RSS) to intravenous injections of adrenaline or noradrenaline or to stimulation of the cut left splanchnic nerve at various frequencies per sec for 180 shocks. Time marker, minutes.

450 shocks were delivered at frequencies ranging from 3·5 to 320/sec, to compare the response at very short stimulus intervals with longer intervals in one experiment: the various stimulations were delivered in a random order. This confirmed that at 30/sec stimulation output is maximal, and that it falls off sharply at faster rates. The interesting observation was made, however, that at 350/sec output recovered somewhat. It appeared, in short, that two separate processes in the response to successive shocks were occurring: the first on a short time scale, lasting only 10 msec or less; the second much slower, with a peak around 30 msec, and lasting up to 300

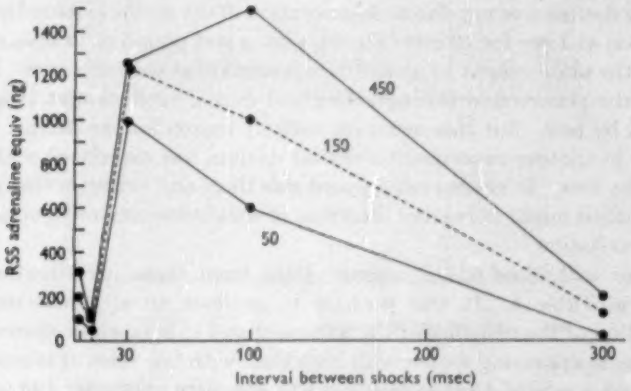



*Fig. 6.* The relation of the output of sympathetic amines (measured as adrenaline equivalents on the blood-bathed isolated rat stomach strip) to the interval between shocks given to the cut left splanchnic nerve. Upper line = 450 shocks; middle line = 150 shocks; lower line = 50 shocks.

or 1000 msec. The position of the peak is not constant, but varies with the number of shocks given, tending to a lower frequency as the volleys delivered increase.

*Output with constant duration of stimulation*

In two experiments, continued stimulation of the splanchnic nerve at various rates with rather brief rest periods was associated with decline of output at excitation rates above 8/sec. But very large outputs might occur with stimulation at 8/sec or higher for a sustained period, if generous rest intervals were allowed. Thus in one experiment stimulation at 10/sec for 10 min produced a total of 36·5 $\mu$g combined amine, and a total of 38·0 $\mu$g was produced by excitation at 16/sec for 10 min. It was thus clear that with these longer periods of excitation output did not simply depend on rate of excitation, but that a phenomenon resembling 'fatigue' of the synapse

could be produced. Experiments were therefore made to produce it deliberately and to determine the factors governing its appearance.

*Splanchnic nerve fatigue*

*Assays of collected adrenal venous blood.* In two cats splanchnic nerve stimulation at progressively raised frequencies but with only brief rest intervals was found to cause a fall in output of noradrenaline and adrenaline into the adrenal venous blood when the rate of excitation, over a period of 8–10 min, exceeded 8–16/sec. In the first experiment the combined amine output at 16/sec was little more than a tenth of that at 8/sec. But this decline was not due to deterioration of the nerve, since subsequent excitation at 1/sec for 10 min (Fig. 7), after a rest period of 14 min, elicited almost the same output as an earlier stimulation at the same rate. In this animal the plasma flow through the gland during excitation at 16/sec was reduced by half. But this seems an unlikely reason for the decline in output, for in another experiment a similar decline was associated with a rise in plasma flow. In neither experiment was there any evidence that output of adrenaline might fail earlier than that of noradrenaline during splanchnic nerve excitation.

*Assays with blood-bathed organs.* Data from these investigations are shown in Table 6. It was possible to produce an apparent complete exhaustion of the response of the adrenal gland to splanchnic stimulation, this fatigue appearing sooner with high than with low rates of stimulation. Thus with a rate of 4/sec exhaustion was complete only after 193 min. In another animal no signs of fatigue were apparent after stimulation at 5/sec for 35 min, but exhaustion occurred after 50 min with stimulation at 8/sec, within 20 min at 16/sec, and 14 min at 32/sec. In another experiment only partial exhaustion was found after 60 min at 16/sec, while in yet another cat no exhaustion was seen when stimulating for 80 min at 10/sec. In two other animals stimulating at a rate of 32/sec led to complete exhaustion within 25 and 60 min respectively.

In all these experiments exhaustion was considered to have occurred when the blood-bathed assay tissue had regained its original tone. This return of tone during continuous splanchnic stimulation was not due to a developing insensitivity of the assay tissues to circulating sympathins as found in the experiments of Feldberg, Minz & Tsudzimura (1934); indeed, they usually became more sensitive to adrenaline, noradrenaline and isopropylnoradrenaline as the experiment progressed. Figure 8 illustrates how adrenaline and noradrenaline are still fully effective on the rat stomach strip at the time when tone had fully returned in the face of continuing splanchnic excitation.

That complete depletion of the sympathetic amines within the adrenal

gland was not the explanation of the unresponsiveness of the gland to nerve stimulation could be demonstrated by the intravenous injection of 0·25–0·5 mg nicotine. This was followed by prompt maximal relaxation of the assay tissues. Nicotine injected in 1/15th–1/12th of this dose into the polythene tubing leading to the blood-bathed assay tissues had no direct effect on the rat stomach strip; it relaxed the chick rectum slightly but

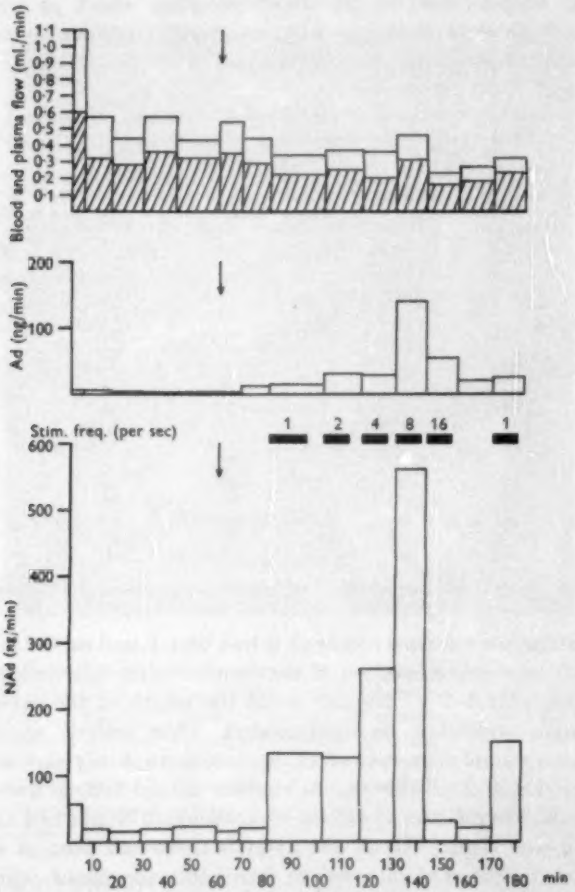



Fig. 7. The outputs of adrenaline and noradrenaline in the suprarenal venous blood before and after section of the left splanchnic nerve (downward pointing arrow) and in association with stimulation of the cut splanchnic nerve at varying frequencies. ■, duration of stimulation, 15 or 10 min. Frequencies of stimulation, reading left to right: 1, 2, 4, 8, 16 and 1 per sec. □, suprarenal venous blood flow, ▨, suprarenal plasma flow.

much less than did the injection of the larger dose of nicotine into the whole animal. The proportionate dose (1/15th–1/12th) was calculated on the basis of a blood volume in the cats of 120–150 ml. and a volume of extracorporeal blood of 10 ml. in the organ bath and connecting tubing.

The exhaustion of the response to nerve stimulation could not be due to electrode polarization (see Methods); nor was it due to the stimulus becoming submaximal by the short-circuiting effect of accumulating tissue fluid, since when fatigue was established doubling the voltage produced no significant alteration in response of the assay tissues. Moreover

TABLE 6. Exhaustion after prolonged splanchnic stimulation: blood-bathed organ experiments. (RSS = rat stomach strip; CR = chick rectum; RD = rat duodenum)

| Expt. no. | Interval before stimulation (min)* | Stimulation: Rate (per sec) | Stimulation: Duration (min) | Assay tissues | Time (min) of complete return to resting level during continuous stimulation: Assay tissues | Blood pressure |
|---|---|---|---|---|---|---|
| 18 | 310 | 16 | 60 | RSS | 60 (50 % return) | 15 |
| 21 | 180 | 32 | 46 | RSS | 25 | 4 |
| | | | | CR | 10 | — |
| 22 | 75 | 32 | 33 | RSS | 14 | 15 |
| | | | | CR | 12 | — |
| | 230 | 16 | 37 | RSS | 20 | 11 |
| | | | | CR | 9 | — |
| | 290 | 8 | 63 | RSS | 50 | 17 |
| | | | | CR | 15 | — |
| | 370 | 5 | 35 | RSS | No fatigue | 9 |
| | | | | CR | — | — |
| 23 | 96 | 4 | 201 | RSS | 193 | 35 |
| | | | | CR | 35 | — |
| 24 | 332 | 10 | 80 | RSS | No fatigue | 27 |
| | | | | RD | — | — |
| 31 | 40 | 32 | 60 | RSS | 60 | 26 |

* Interval between completion of the preparation and beginning stimulation.

20 V stimulation was used although it had been found earlier in the experiment that maximal relaxation of the stomach strip followed nerve stimulation with only 1–2 V. Neither could the length of the experiment nor the animals condition be incriminated. Thus fatigue appeared more rapidly at a stimulation rate of 32/sec, but more slowly at 8/sec some 215 min later (Expt. 22, Table 6). In another animal fatigue was incomplete after 60 min stimulation at 16/sec, even though 370 min had elapsed since recording was begun. In all the animals there had been at most an insignificant decline of blood pressure during the experiment, and splanchnic nerve stimulation invariably produced a substantial rise of blood pressure (50–140 mm Hg). The blood pressure immediately preceding stimulation was never lower than 60 mm Hg and in the majority of animals lay between 90 and 180 mm Hg.

Evidence was obtained that output of adrenaline failed sooner than that

of noradrenaline during nerve stimulation. The chick rectum regained normal tone before the blood-bathed rat stomach strip during prolonged splanchnic stimulation (Figs. 8, 9) although it is much more sensitive to adrenaline than to noradrenaline, whereas the rat stomach strip is only about twice as sensitive to adrenaline as to noradrenaline. This pheno-

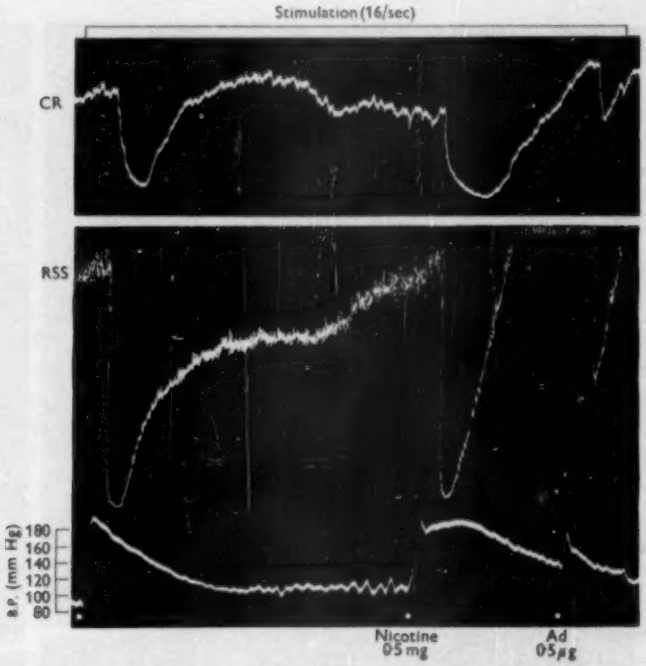



**Fig. 8.** Cat, 2·2 kg; chloralose. The response of the blood-bathed isolated chick rectum (CR) and rat stomach strip (RSS) and the blood pressure (B.P.) to prolonged stimulation at a frequency of 16/sec of the cut left splanchnic nerve. Note the early adaptation of the blood pressure to circulating sympathetic amines coinciding with the return of tone to the chick rectum, and the eventual return of tone to rat stomach strip. Considerable relaxations of both blood-bathed tissues following the intravenous injection of either 0·5 mg nicotine or 0·5 μg adrenaline. Time marker, minutes.

menon was seen whether the rate of excitation was 16/sec (Fig. 8) or 4/sec (Fig. 9). The result might only mean that the chick rectum has a greater facility to regain tone than the other assay tissues. This is excluded, however, by the fact that the relaxation of the chick rectum to adrenaline may follow the same time course as the stomach strip (Fig. 8) or may be somewhat slower (Fig. 3).

much less than did the injection of the larger dose of nicotine into the whole animal. The proportionate dose (1/15th–1/12th) was calculated on the basis of a blood volume in the cats of 120–150 ml. and a volume of extracorporeal blood of 10 ml. in the organ bath and connecting tubing.

The exhaustion of the response to nerve stimulation could not be due to electrode polarization (see Methods); nor was it due to the stimulus becoming submaximal by the short-circuiting effect of accumulating tissue fluid, since when fatigue was established doubling the voltage produced no significant alteration in response of the assay tissues. Moreover

TABLE 6. Exhaustion after prolonged splanchnic stimulation: blood-bathed organ experiments. (RSS = rat stomach strip; CR = chick rectum; RD = rat duodenum)

| Expt. no. | Interval before stimulation (min)* | Stimulation: Rate (per sec) | Stimulation: Duration (min) | Assay tissues | Time (min) of complete return to resting level during continuous stimulation: Assay tissues | Blood pressure |
|---|---|---|---|---|---|---|
| 18 | 310 | 16 | 60 | RSS | 60 (50 % return) | 15 |
| 21 | 180 | 32 | 46 | RSS | 25 | 4 |
| | | | | CR | 10 | — |
| 22 | 75 | 32 | 33 | RSS | 14 | 15 |
| | | | | CR | 12 | — |
| | 230 | 16 | 37 | RSS | 20 | 11 |
| | | | | CR | 9 | — |
| | 290 | 8 | 63 | RSS | 50 | 17 |
| | | | | CR | 15 | — |
| | 370 | 5 | 35 | RSS | No fatigue | 9 |
| | | | | CR | — | — |
| 23 | 96 | 4 | 201 | RSS | 193 | 35 |
| | | | | CR | 35 | — |
| 24 | 332 | 10 | 80 | RSS | No fatigue | 27 |
| | | | | RD | — | — |
| 31 | 40 | 32 | 60 | RSS | 60 | 26 |

* Interval between completion of the preparation and beginning stimulation.

20 V stimulation was used although it had been found earlier in the experiment that maximal relaxation of the stomach strip followed nerve stimulation with only 1–2 V. Neither could the length of the experiment nor the animals condition be incriminated. Thus fatigue appeared more rapidly at a stimulation rate of 32/sec, but more slowly at 8/sec some 215 min later (Expt. 22, Table 6). In another animal fatigue was incomplete after 60 min stimulation at 16/sec, even though 370 min had elapsed since recording was begun. In all the animals there had been at most an insignificant decline of blood pressure during the experiment, and splanchnic nerve stimulation invariably produced a substantial rise of blood pressure (50–140 mm Hg). The blood pressure immediately preceding stimulation was never lower than 60 mm Hg and in the majority of animals lay between 90 and 180 mm Hg.

Evidence was obtained that output of adrenaline failed sooner than that

of noradrenaline during nerve stimulation. The chick rectum regained normal tone before the blood-bathed rat stomach strip during prolonged splanchnic stimulation (Figs. 8, 9) although it is much more sensitive to adrenaline than to noradrenaline, whereas the rat stomach strip is only about twice as sensitive to adrenaline as to noradrenaline. This pheno-

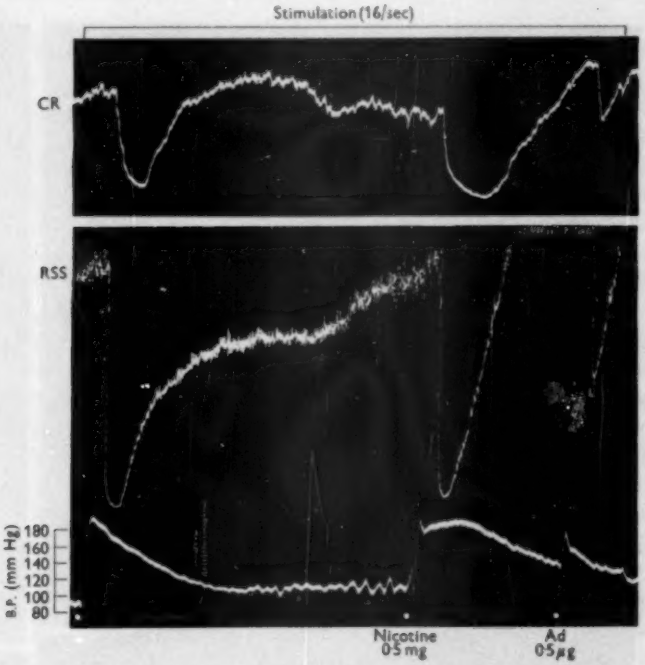



Fig. 8. Cat, 2·2 kg; chloralose. The response of the blood-bathed isolated chick rectum (CR) and rat stomach strip (RSS) and the blood pressure (B.P.) to prolonged stimulation at a frequency of 16/sec of the cut left splanchnic nerve. Note the early adaptation of the blood pressure to circulating sympathetic amines coinciding with the return of tone to the chick rectum, and the eventual return of tone to rat stomach strip. Considerable relaxations of both blood-bathed tissues following the intravenous injection of either 0·5 mg nicotine or 0·5 μg adrenaline. Time marker, minutes.

menon was seen whether the rate of excitation was 16/sec (Fig. 8) or 4/sec (Fig. 9). The result might only mean that the chick rectum has a greater facility to regain tone than the other assay tissues. This is excluded, however, by the fact that the relaxation of the chick rectum to adrenaline may follow the same time course as the stomach strip (Fig. 8) or may be somewhat slower (Fig. 3).

*Adaptation to sympathetic amines*

In the experiments on suprarenal exhaustion it was noticed (Table 6) that the cat's blood pressure returned to control levels considerably earlier than the rat stomach strip, and at about the same time as the chick

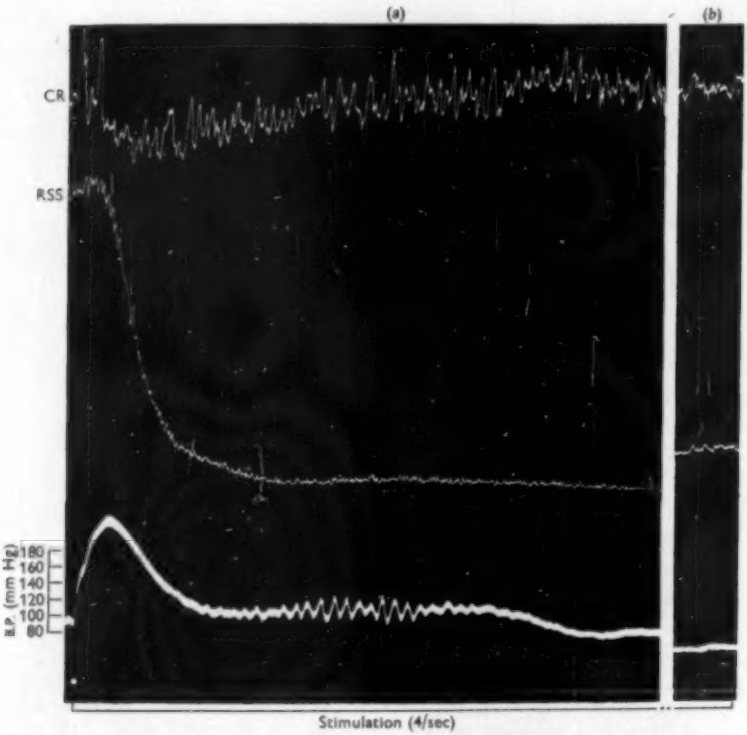



Fig. 9. Cat, 3·5 kg; chloralose. Effect of prolonged stimulation at a frequency of 4/sec of the cut left splanchnic nerve on the response of the blood-bathed isolated chick rectum (CR) and rat stomach strip (RSS) and of the blood pressure (B.P.). Note the early adaptation of the blood pressure and return of tone to the blood-bathed isolated chick rectum (*a*), while the rat stomach strip remains relaxed 125 min after commencing stimulation (*b*). Time marker, minutes.

rectum. Unlike the chick rectum, the blood pressure is more sensitive to noradrenaline than to adrenaline, so that a rising proportion of noradrenaline cannot account for the blood-pressure recovery. It suggests that there may be a difference in the manner in which various tissues accommodate to the effect of these amines. This was tested in four experi-

ments by infusing noradrenaline, adrenaline, or a mixture of these in equal parts, while recording the blood pressure and the responses of the blood-bathed stomach strip and chick rectum. In all cases the blood pressure returned to normal while the stomach strip was still fully relaxed; in only one experiment, with a mixture of noradrenaline and adrenaline, was there a partial recovery of tone with the chick rectum.

*Effect of ganglion-blocking drugs on the splanchnic-suprarenal synapse*

The experiments described above were undertaken to define the behaviour of the splanchnic-suprarenal synapse under various conditions of excitation. Once established, this knowledge could be applied to examining quantitatively the susceptibility of the synapse to blocking agents. Although a good deal of knowledge exists about the paralysis of homologous autonomic ganglia, our information about paralysis of the adrenal has advanced little since the study by Feldberg *et al.* (1934) of the effects of nicotine and atropine.

In early experiments ganglion-blocking drugs were tested during intermittent splanchnic stimulation at 10/sec for 30 sec; but the large relaxation produced by this excitation was little altered, for instance, by intravenous injections of hexamethonium up to 200 $\mu$g/kg. Larger doses (0·5 mg/kg) had a good blocking effect, but since other actions in the body became prominent, a more sensitive test seemed desirable. A better method was to test the action of the drugs by determining their effect on the tone of the blood-bathed assay tissues during sustained splanchnic stimulation at low frequencies (3–5/sec), which could be maintained for long periods without nerve exhaustion supervening; this stimulation rate produced a half maximal relaxation of the stomach strip. Provided the drug has no direct action on the strip, a paralysis of transmission shows itself by a recovery of tone in the strip and the relaxation re-establishes itself as the effect of the ganglion-blocking agents wears off.

Hexamethonium ($C_6$) was found to be the most potent of the ganglion-blocking members of the methonium series tested, with $C_5$, $C_7$ and $C_4$ in descending order of potency (50, 6·25 and 2·3 % respectively of the potency of $C_6$). $C_6$ was about five times as potent as tetraethyl ammonium bromide. The recovery of tone produced by, for example, 200 $\mu$g $C_6$ or 1 mg TEA/kg was incomplete and lasted about 7–10 min.

A proportion of the total dose (1/15th) of these drugs, injected into the tubing in series between the cannulated carotid artery and the bath containing the blood-bathed stomach strip, did not produce any significant effect on the assay tissues. It was impossible, however, to determine the relative potencies of tubocurarine or probanthine, as these had a direct

atropine-like relaxing effect on the stomach strip, which masked the possible recovery in tone looked for.

The similarity to autonomic ganglia showed itself in two other aspects. First, the synapse was resistant to decamethonium, which in a dose of 10 mg/kg had no effect on the response of the stomach strip to splanchnic nerve excitation. Secondly, the synapse was first excited and later blocked by nicotine.

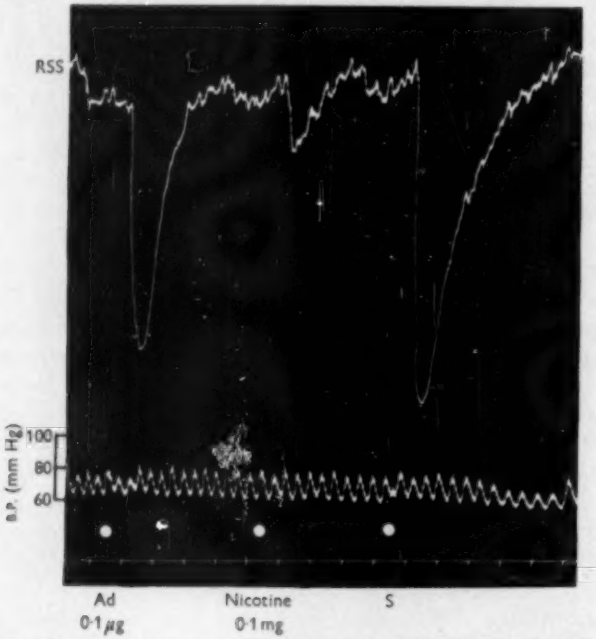



Fig. 10. Cat, 3·0 kg chloralose. The response of the blood-bathed isolated rat stomach strip (RSS) to intravenous injections of 0·1 μg adrenaline, 0·1 mg of nicotine or to stimulation of the cut left splanchnic nerve (S) at a frequency of 10/sec (100 shocks). Time marker, minutes.

*Nicotine.* The stimulant effect of nicotine on the adrenal medulla is well known, and nicotine in large doses has often been used to reduce or remove the response to splanchnic stimulation (Feldberg *et al.* 1934). Both these effects can be readily demonstrated with the blood-bathed stomach strip. It proved a very sensitive indicator of the stimulant action of nicotine on the medulla. Feldberg *et al.* required 0·1 mg given by arterial injection close to the gland to produce a good effect on the blood pressure. But with the stomach strip the same dose intravenously produced a good relaxation, the blood pressure being unaffected (Fig. 10). If the dose was

raised to 2 mg a slight reduction of the effect of splanchnic stimulation sometimes occurred. With repeated doses of 2 mg every 3 min up to a total of 12 mg a dwindling response was recorded (Fig. 11), and in the end splanchnic stimulation was greatly reduced.

The question arose, which appears not to have been previously considered, as to how far depletion of amine stores contributed to the fading response to stimulation. Two tests were applied. First, the release of amine to a fixed dose of histamine was tested before and after nicotine (the strip is exceedingly resistant to histamine). The release of amine by histamine was reduced by nicotine, but less so than the release to nerve stimulation

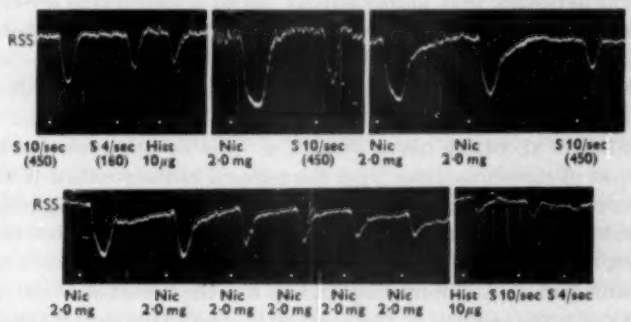



Fig. 11. Cat, 3·6 kg; chloralose. Effect of single or serial doses of nicotine (Nic) on the response of the blood-bathed isolated rat stomach strip (R88) to splanchnic stimulation (S) at frequencies of 10/sec (total of 450 shocks) or of 4/sec (180 shocks) and to 10 μg histamine (Hist). Time marker, minutes.

(Fig. 11). If a massive amount of nicotine was given in divided doses in a relatively short time, complete abolition of nerve response occurred; the histamine response was reduced but still present. Secondly, it seemed improbable from our experiments on exhaustion that depletion could account by itself for the abolition of the response to nerve stimulation. This was tested directly in an experiment in which a series of brief nerve stimulations was substituted for the repeated doses of nicotine, producing relaxations of the strip comparable to those produced by the nicotine; no decline in response to splanchnic stimulation was found. It appears that the effect of nicotine on the adrenal synapse is a true synaptic block, although the reduced response to histamine may imply some contribution by amine depletion.

Since nicotine applied in sufficient doses directly to the strip may excite it, the relaxant effect could be somewhat underestimated. We believe, however, that the stimulant action of nicotine in the doses used, by the intravenous route, is virtually negligible. There is a circulatory latent

period of 1–2 min before the strip relaxes in response to a discharge of amines from the medulla; we have never seen any initial stimulant effect on the strip during this period, although it is then that the carotid blood contains nicotine at its highest concentration. As an additional control we have tested nicotine by injection into the extracorporeal circuit, in the same fraction of the intravenous dose as the ratio of the blood volumes of the circuit and the animal; such doses or slightly higher ones are ineffective.

## DISCUSSION

Although early experiments on adrenal denervation (Stewart & Rogoff, 1916, 1917) indicated that amine output fell to undetectable levels, subsequent workers (Vogt, 1952; Duner, 1953; Rapela, 1956) agree that there is a constant small release of catechols from either the acutely or chronically denervated adrenal. Our results, restricted to the effects of acute nerve section, agree with those previously reported. In the normal cat under chloralose there is, before nerve section, a variable but sometimes quite large output of catecholamines from the adrenal (noradrenaline 3–198 ng/min, mean 54·5 ng/min; adrenaline < 1–209 ng/min, mean 36·2 ng/min), and under pentobarbitone similar figures were found. Nerve section reduced the output of both amines on an average by 60–70 %, sometimes to almost undetectable levels. An interesting finding was the subsequent rise in output after the nerve section, in the absence of nerve stimulation, or of any other obvious stimulus to secretion. We have concluded that before nerve section the stores of amine in the gland are slightly depleted by the normal activity of the splanchnic nerve; and that when this drain on the stores is prevented, amine accumulates until a spontaneous 'overspill' occurs.

When the splanchnic nerve is stimulated, there is a distinct 'threshold' frequency which the excitation must exceed for output to be raised. If duration of stimulation is unlimited then the rate, for a consistent response, must be 2–4 shocks/sec or more. If the optimal rate of excitation is used (about 30/sec) then something like 20 shocks are needed to increase output detectably. These properties are quite unlike those of, say, the nictitating membrane or neuromuscular junction, where single shocks produce distinct responses. It implies that, in the development of reflex activity by the adrenals, a more intense nervous discharge is called for from the splanchnic nerve than in the presynaptic fibres of other junctions. With effective nerve stimulation, output depended critically on the interval between successive shocks applied. As the interval was made shorter, with a constant number of shocks, output rose to a peak at about 30 msec intervals (stimulation rate 30/sec). Then output declined again, with more rapid shocks, to a minimum at about 10 msec interval. Finally, some degree of recovery occurred with stimulus interval made still shorter.

A number of mechanisms may be involved in this pattern of response. First must be considered a summation between the effects of acetylcholine released by successive shocks at the splanchnic–medullary-cell synapse. The wide distribution of cholinesterase (which also occurs in the medulla) and the invariable transience of action of acetylcholine exclude its direct participation in any prolonged response. But a process, capable of temporal summation, complete within 10 msec would be not unreasonable; so that the greater output at 3 msec intervals compared with 10 msec intervals could be attributed to persistence of the transmitter or of its immediate electrical effect for a few milliseconds.

Secondly, one might postulate an excitatory state, set up in the gland cell by the transmitter, which, if sufficiently intense, leads to a discharge of amines. The fact that single shocks are ineffective, and that it needs, under the best conditions, about 20 shocks to produce a detectable response, indicates that the excitatory state evoked by a single volley is well below the threshold for amine discharge. If, then, shocks are repeated at sufficiently short intervals, this threshold will eventually be exceeded; and the magnitude of the total excitatory state achieved will be a measure (not necessarily linear) of the slowness of decline of excitatory state after each shock. Such a model will account for a rising output with shortening stimulus interval; but it does not account for the decline of output when the interval decreases from 30 msec, unless it is also supposed that during the rise of the excitatory state, or for some period after its start, the medullary cell is relatively refractory to additional excitation.

A third interpretation is as follows. First, the decline of output with shocks less frequent than 10–30/sec might be attributed to some process of local uptake by the gland, like that proposed for the spleen by Brown & Gillespie (1957), who found that the noradrenaline output from the splenic nerves wanes in a similar way. The situation here, however, is somewhat different. In the spleen the receptor tissue is in direct relation to the nerves, and uptake or destruction of the amines by splenic tissue is a plausible supposition. In the adrenal, however, the final receptor is the body as a whole; if, as in our experiments, adrenal venous blood is collected, the only tissue available to take up the amines released is the blood in the gland, the supporting tissues of the medulla, and the medullary cells themselves. Since the cells can regain their amine content after its exhaustion, a reabsorption by the medullary cells of amine secreted a moment earlier deserves consideration. Secondly, the decline of output with shocks more frequent than 30/sec could be attributed to a process of synaptic fatigue or, possibly, to a failure of nerve-terminal conduction at high rates.

The higher rates of output we have obtained, between 3·6 and 3·8 $\mu$g/min for a period of 10 min stimulation at 10 or 16/sec, are similar to those

found by earlier workers. The work by Eade & Wood (1958) is most similar in method to our own; they found that excitation at 20/sec for 4·75 min released 5·15 $\mu$g/min (average) of catechol amines into the adrenal venous blood. Put into relation with the figures provided by Butterworth & Mann (1957) for the amine content of cat's adrenals (range 178–992 $\mu$g), mean *ca.* 320 $\mu$g/gland), this means that between 1 and 2 % of the medullary contents can be released per minute. Such a figure implies, further, that exhaustion of the release for maximal rates of output should occur within 50–100 min at most, unless resynthesis or re-storage takes place.

Our experiments with continued excitation show that 'fatigue' in fact sets in considerably earlier than this, especially with the faster rates of stimulation. Thus, at 32/sec fatigue was sometimes quite rapid, being complete in two experiments in 14 and 25 min; at 4/sec, where the output is of the order of 0·2 % of gland content per minute, it required 193 min stimulation to exhaust the response. This in itself points to the existence of fatigue due to some cause other than depletion of the stores. This was confirmed by finding that after the response to continued nerve stimulation had failed nicotine could still elicit a vigorous discharge of amines. The fatigue, therefore, is presumably not post-synaptic in nature.

There is no information as to the physiological rate of nervous discharge in the splanchnic nerves. The properties of the synapse would allow it to work effectively up to rates of 30–100/sec, since increasing outputs are obtained with increase of rate up to this level. According to Folkow (1952), post-ganglionic sympathetic nerves to a limb do not fire faster than 6–8/sec; but these are performing a quite different function.

Previous workers have paid considerable attention to the proportion of noradrenaline to adrenaline released by nervous excitation. Thus Rapela (1956) reported that at a high stimulation rate a selective increase of adrenaline release occurred. In general, we have found that with brief periods of excitation the proportion of the amines did not vary with rate in any consistent way. But in one experiment excitation at 512/sec did produce an increase of adrenaline release only. More important is the question of a changing amine ratio during prolonged excitation. Bülbring & Burn (1949) found that in five out of nine experiments with repeated intermittent stimulation the adrenaline proportion fell. On the other hand, Lund (1951) and Outschoorn (1952) concluded that the amine ratio was unchanged. It is worth noting that, in any case, the amine ratio of cat's adrenal glands is extremely variable, ranging from 13·3 to 90·7 % noradrenaline (Butterworth & Mann, 1957). In our experiments with blood-bathed tissues there was a consistent tendency during prolonged excitation of the splanchnic nerve for the chick rectum (specifically sensitive to adrenaline) to regain its tone before the rat stomach strip (approxi-

mately equally sensitive to both amines), so that our evidence supports the conclusion reached by Bülbring & Burn.

During these experiments the observation was also made that the blood pressure returned to its control level much more quickly than did the rat stomach strip. Since noradrenaline is more effective than adrenaline on the blood pressure, a change in amine ratio does not account for this. The observation prompted experiments on the accommodation of blood pressure and of the blood-bathed tissues to infusions of amines. This showed that the blood pressure would always accommodate, completely if time were allowed, although the rat stomach strip would never do so. An interesting parallel finding is the record, in the experiments reported by Blacket, Pickering & Wilson (1950), that rabbits which had become tolerant to the pressor effect of noradrenaline or adrenaline infusions had grossly distended viscera. If accommodation to the excitatory but not to the relaxant effects of these amines is possible, an explanation of adrenaline or noradrenaline 'shock' immediately appears, that this is due to the unopposed vasodilator effect of these drugs after tolerance has developed to the vasoconstriction.

Finally, the possibility was explored in our experiments that the splanchnic-adrenal synapse might have a specific sensitivity to blocking agents, such that drugs might be found to paralyse it selectively, just as hexamethonium or decamethonium can be used at the ganglionic or neuromuscular synapses. By three tests, however, it resembled the autonomic ganglia with which it is homologous. First it was paralysed, better by hexamethonium than by its $C_4$, $C_5$, or $C_7$ homologues. As with autonomic ganglia, block was produced more readily during sustained excitation than with brief bursts of excitation. Secondly, decamethonium was inactive. Thirdly, nicotine initially excited and then paralysed the synapses. Such a response is typical of ganglia, but unlike that of cat's voluntary muscle, on which nicotine is almost devoid of competitive blocking action (W. D. M. Paton & E. C. Savini, unpublished). It is clear from the experiments by Feldberg *et al.* (1934) that the sensitivity of the synapse to block of an atropinic kind is rather small, and may, indeed, be not larger than that now known to exist at the autonomic ganglia. A selective blocking agent for the adrenal synapse, sparing the autonomic ganglia, would be of great interest and usefulness; but it seems that the faithfulness of the gland to its autonomic ancestry stands in the way of such a development.

## SUMMARY

1. The output of sympathetic amines from the cat adrenal gland in response to nerve stimulation was studied both by assay of the amines in adrenal venous blood, and by their effect on isolated assay tissues

(rat stomach strip, chick rectum) included within an extracorporeal circulation.

2. The resting output of sympathetic amines (particularly noradrenaline) falls after acute splanchnic-nerve section, but rises again in the absence of nerve stimulation.

3. There is a threshold stimulation frequency for augmented amine release; thus excitation of the cut splanchnic nerve at 1/sec has virtually no effect unless prolonged for about 15 min; output is invariably raised with stimulation at a rate of 4/sec.

4. With adequate rest intervals output increases approximately linearly with increasing frequency of stimulation up to a rate of 30–60 sec, after which output declines. At such rates about 60 shocks are needed to increase output significantly; in one experiment 13 shocks were effective. Increased output above control values may be found with stimulation rates as high as 500/sec, given in a brief burst lasting 100 msec. It appears that two separate processes of interaction occur between successive shocks: the first on a short time scale lasting only 10 msec or less, the second much slower, with a peak at 30 msec, and lasting for 300–1000 msec.

5. Fatigue of the response occurs if stimulation is either protracted (even at excitation rates as low as 4/sec) or if inadequate rest periods are allowed between bursts of 10 min stimulation at rates of 4–32/sec; this appears to be a synaptic fatigue. During prolonged stimulation, the output of adrenaline declined before that of noradrenaline.

6. During a prolonged output of amines in response to splanchnic nerve stimulation, or during an infusion of noradrenaline or adrenaline, the pressor blood-pressure response accommodates, but the relaxant response of the stomach strip does not.

7. Ganglion-blocking drugs of the methonium series paralyse the adrenal synapse, especially during sustained stimulation. A range of drugs was tested to see whether a selective blocking agent was likely to exist; but none was found. The splanchnic-adrenal synapse appears to have the properties of autonomic ganglia with which it is homologous.

This work was carried out during the tenure by one of us (E.M.) of a Medical Research Council Clinical Research Fellowship. We are indebted to Mr D. A. Green and his staff for technical assistance.

## REFERENCES

Barsoum, G. S. & Gaddum, J. H. (1935). The pharmacological estimation of adenosine and histamine in blood. *J. Physiol.* **85**, 1–14.

Biedl, A. (1897). Die Innervation der Nebennieren. *Pflüg. Arch. ges. Physiol.* **47**, 443–483.

Blacket, R. B., Pickering, G. W. & Wilson, G. M. (1950). The effect of prolonged infusions of noradrenaline and adrenaline on the arterial pressure of the rabbit. *Clin. Sci.* **9**, 247–257.

Brown, G. L. & Gillespie, J. S. (1957). The output of sympathetic transmitter from the spleen of the cat. *J. Physiol.* **138**, 81–102.

BÜLBRING, E. & BURN, J. H. (1949). Liberation of noradrenaline from the suprarenal gland. *Brit. J. Pharmacol.* **4**, 202–208.

BUTTERWORTH, K. R. & MANN, M. (1957). A quantitative comparison of the sympathomimetic amine content of the left and right adrenal glands of the cat. *J. Physiol.* **136**, 294–299.

DE JALON, P. G., BAYO, J. B. & DE JALON, M. G. (1945). Sensible y nuevo método de valoración de adrenalina en útero aislado de rata. *Farmacoterap. act.* **2**, 313–318.

DREYER, G. P. (1899). On secretory nerves to the suprarenal capsules. *Amer. J. Physiol.* **2**, 203–219.

DUNER, H. (1953). The influence of the blood glucose level on the secretion of adrenaline and noradrenaline from the suprarenal. *Acta physiol. scand.* **28**, Suppl. 102.

EADE, N. R. & WOOD, D. R. (1958). The release of adrenaline and noradrenaline from the adrenal medulla of the cat during splanchnic stimulation. *Brit. J. Pharmacol.* **13**, 390–394.

ELLIOTT, T. R. (1912). The control of the suprarenal glands by the splanchnic nerves. *J. Physiol.* **44**, 374–409.

ELLIOTT, T. R. (1913). The innervation of the adrenal glands. *J. Physiol.* **46**, 285–290.

FELDBERG, W., MINZ, B. & TSUDZIMURA, H. (1934). The mechanism of the nervous discharge of adrenaline. *J. Physiol.* **81**, 286–304.

FOLKOW, B. (1952). Impulse frequency in sympathetic vasomotor fibres correlated to the release and elimination of the transmitter. *Acta physiol. scand.* **25**, 49–76.

GADDUM, J. H., PEART, W. S. & VOGT, M. (1949). The estimation of adrenaline and allied substances in the blood. *J. Physiol.* **108**, 476–481.

GARRY, R. C. & GILLESPIE, J. S. (1955). The responses of the musculature of the colon of the rabbit to stimulation, *in vitro*, of the parasympathetic and of the sympathetic outflows. *J. Physiol.* **128**, 557–576.

LUND, A. (1951). Release of adrenaline and noradrenaline from the suprarenal gland. *Acta pharm. tox., Kbh.*, **7**, 309–320.

MALMEJAC, J. & NEVERRE, G. (1950). Action of chloralose on adrenaline section. *C.R. Soc. Biol., Paris*, **144**, 1034–5.

OUTSCHOORN, A. S. (1952). The hormones of the adrenal medulla and their release. *Brit. J. Pharmacol.* **7**, 605–615.

PATON, W. D. M. (1957). A pendulum auxotonic lever. *J. Physiol.* **137**, 35–36*P*.

RAPELA, C. E. (1956). Differential secretion of adrenaline and noradrenaline. *Acta physiol. latinoamer.* **6**, 1–14.

SHIPLEY, R. E. & TILDEN, J. H. (1947). A pithed rat preparation suitable for assaying pressor substances. *Proc. exp. Biol., N.Y.*, **64**, 453–455.

STEWART, G. N. & ROGOFF, J. M. (1916). The spontaneous liberation of epinephrin from the adrenals. *J. Pharmacol.* **8**, 479–524.

STEWART, G. N. & ROGOFF, J. M. (1917). Quantitative experiments on the liberation of epinephrin from the adrenals after section of their nerves, with special reference to the question of the indispensability of epinephrin for the organism. *J. Pharmacol.* **10**, 1–48.

TSCHEBOKSAROFF, M. (1911). Ueber sekretorische Nerven der Nebennieren. *Pflüg. Arch. ges. Physiol.* **137**, 59–122.

VANE, J. R. (1957). A sensitive method for the assay of 5-hydroxytryptamine. *Brit. J. Pharmacol.* **12**, 344–349.

VANE, J. R. (1958). The blood-bathed isolated organ: a method for testing the circulating blood for active substances. *J. Physiol.* **143**, 75–76*P*.

VOGT, M. (1952). The secretion of the denervated adrenal medulla of the cat. *Brit. J. Pharmacol.* **7**, 325–330.

WEST, G. B. (1950). Liberation of adrenaline from the suprarenal gland of the rabbit. *Brit. J. Pharmacol.* **5**, 542–548.

YOUNG, J. Z. (1939). Partial degeneration of the nerve supply of the adrenal. A study in autonomic innervation. *J. Anat., Lond.*, **73**, 540–550.

*J. Physiol.* (1961), **155**, *pp.* 28–45
*With 3 text-figures*
*Printed in Great Britain*

# THE OSMOTIC COEFFICIENTS OF HAEMOGLOBIN IN RED CELLS UNDER VARYING CONDITIONS

By PADDY DEBORAH McCONAGHEY and M. MAIZELS
*From the Department of Clinical Pathology, University College Hospital, London, W.C.* 1

(*Received* 17 *June* 1960)

An osmolal solution in water may be defined as one whose freezing point is 1·86° C below that of water; such a solution is said to contain 1 osmole of solute in 1 kg water. In ideal systems an osmolal solution is also molal, and in the case of some non-electrolytes of small molecular size this ideal relation is virtually attained: thus lactose in 0·01 molal solution gives a molal depression of freezing point of 1·86° C, while with a 0·2 molal solution the molal depression is 1·88° C; hence the osmotic coefficient, that is the ratio of osmoles to moles, is effectively unity. Many other substances, however, do not exhibit this simple relation, and in these instances one osmole corresponds to the number of moles of the substance which, if added to 1 kg water, would depress the freezing point by 1·86° C. In the case of solutions of macromolecules the osmotic coefficient may greatly exceed unity. Thus Adair (1929) has found that the osmotic pressure of haemoglobin solutions is greater than would be expected from the molarity. He shows that in a solution containing 2 mM haemoglobin the osmolarity is not 0·002 but 0·003, the osmotic coefficient being 1·5; while the osmolarity of a 5 mM solution (5·2 m-molal) is 0·0175 and the osmotic coefficient of the haemoglobin is 3·4. Thus as the concentration rises from 2 to 5·2 m-mole/kg water, the osmotic coefficient increases progressively. There are apparently no figures for the osmotic coefficients of haemoglobin at higher concentrations, and since the movements of haemoglobin molecules are known to become restricted when the concentration exceeds that in normal red cells (Perutz, 1948) it seemed of interest to investigate the osmotic coefficients of haemoglobin at very high concentrations, within the range of increasing restriction of molecular movements.

In the case of the red cell it is possible to do this in two ways. First, when red cells are suspended in a solution of non-electrolyte like lactose, loss of anion, cation and water leads to considerable cell shrinkage, and in these circumstances osmotic coefficients exceeding 10 have been observed (Bolingbroke & Maizels, 1960) so that 13 m-mole haemoglobin in 1 kg water appeared to have an osmolality of 0·1. There was, however, considerable

uncertainty about the precise concentration of haemoglobin, because the space between the centrifuged shrunken and crenated cells is considerable, approaching 30% of the total volume after suspending cells for 3 hr in 10% lactose solution at 37° C, and although in these experiments the amount of lactose in the centrifuged cell mass was estimated, it was not known how much of the lactose was in, on or between the packed cells. In the present paper this uncertainty has been partially remedied by measuring the intercellular (extracellular) space in columns of red cells centrifuged for 30 min at 2000 *g*, with albumin iodinated with $^{131}I$ as marker (Gold & Solomon, 1954; Maizels & Remington, 1959*a*). It has thus been possible to define molal osmotic coefficients approximately for solutions of low salt concentration, containing between 5 and 20 m-mole haemoglobin/kg water. Secondly, when red cells are suspended in hypertonic solutions of sodium chloride they shrink, loss of water being accompanied by little exchange of cation. In these circumstances it is possible to measure osmotic coefficients of haemoglobin in solutions containing a relatively high concentration of salts.

## METHODS

### *Human red cells in lactose solutions*

Heparinized human blood was centrifuged for 10 min and 1 volume of cells suspended in 50 vol. of lactose solution (6 or 10 g/100 ml.). The suspension was rocked for 3 hr at 37° C, and the cells were then separated by centrifuging for half an hour at 2000 *g*, in tubes whose upper portions were wide, and whose lower portions consisted of sealed, graduated and calibrated capillaries holding 0·1 ml. These samples were used for the estimation of Na, K, lactose, intercellular medium and haemoglobin content, each estimation being done in duplicate. pH was measured at 20° C with a glass electrode on a large haemolysed sample. Na and K were estimated with a flame photometer. Further details are given elsewhere (Maizels & Remington, 1959*a*, *b*), but certain points require fuller consideration here.

*Haemoglobin and water content.* It was assumed that the normal fresh red cell contains 34 g haemoglobin and 70 g water/100 ml. Thus if the treated sample *A* contains 48 g haemoglobin/100 ml., its volume is $100 \times 34/48$ or 71% of normal, and its water content (70 − [100–71]/(71) or 57·5 g water/100 ml. Sample *A* may now be used as a standard, and if readings of similar dilutions of *A* and *B* in a photo-electric colorimeter are 300 and 285, the volume of *B* is 74·5% of normal and its water content is 59·5 g/100 ml. In this way one measures not the absolute volume of the treated red cell, but its volume relative to that of the normal cell. (The figures for water content are rounded off to the nearest 0·5%.)

$^{131}I$-*albumin marker.* Difficulty was encountered with some samples of this marker, which retained a proportion of surface-active impurity even after two successive treatments with 10 volumes of washed normal cells (cf. Maizels & Remington, 1959*a*), so that a vessel in which such a sample had been counted might still give 10% of the sample counts even after it had been well washed with water. The container of a satisfactory sample, on the other hand, gave a count after washing which was little more than the background count. It was therefore decided that a suitable sample after treatment with a large excess of red cells to remove surface-active impurities, ought to give a standard value for trapped intercellular medium, when centrifuged with normal cells, of less than 2·2%. The reason for this is as follows: when a solute is added to a suspension of cells, it may be distributed in, on or

between the cells, and that substance which gives the lowest content in centrifuged cells is the one most likely to be limited to the intercellular space. Such a substance is Evans blue, which forms a complex with albumin, the Evans blue-albumin space corresponding to 2% of the packed cell mass (Maizels & Remington, 1959*a*). Evans blue was not used in the present experiments because lactose-treated cells are fragile, and haemoglobin shed during the procedure interferes with the estimation of the Evans blue.

*pH.* The cells were haemolysed in an equal volume of saponin solution (0·06 g/100 ml.) whose pH was adjusted to 7·1; the saponin solution was virtually unbuffered. The pH of this haemolysate agreed closely with the pH of the cells haemolysed by freezing, the differences in three experiments being 0, +0·02 and −0·03 units. It is likely that the pH of haemolysed cells approximates to that of the interior of the intact cells: thus with an external pH of 7·42, the pH of haemolysed cells which had previously been cold-stored and then incubated was 7·26 at 37° C (Harris & Maizels, 1952), while the pH of haemolysed cells from three fresh blood specimens averaged 7·24 (unpublished results); this compares fairly well with Henderson's (1928) figure of about 7·12, calculated from the $CO_2$ and base-bicarbonate concentrations of intact cells.

*Cells in NaCl solution*

Cells were thrice washed in large amounts of NaCl solution to remove $CO_2$ and bicarbonate, and 1 vol. of cells was then suspended in 9 vol. of NaCl solution (0·1–0·45 M) containing 2% serum albumin (for buffering) and also 5 mM-NaF to inhibit residual glycolysis. The pH was adjusted to 6·8, but in spite of the preliminary washing the pH of the suspension rose slightly and 'cell' pH varied between 6·8 and 6·95. The centrifuge tubes used were similar to those already described, but the narrowed stem held 1 ml. instead of only 0·1 ml: the suspension was centrifuged as soon as the adjustment of the pH was completed, and the cells and supernatant analysed.

*Water content.* The centrifuge tube was washed and carefully dried down to the upper face of the red cell column. It was then weighed, dried to constant volume at 105° C, and weighed again. Figures for the water content agreed well with the calculated values based on the haemoglobin content, except in the case of shrunken cells where direct estimation gave figures 1–2% higher than the theoretical values. The reason for this is obscure, but the finding might arise from loss of cell material on shrinking in grossly hypertonic media.

For the estimation of water content, it was thought that 1 ml. cells was necessary to give reasonable accuracy; hence the use of wide-stemmed tubes. But as these tubes only held 10 ml. it was necessary to use a 10–15% cell suspension, and to ensure comparable packing of cells subjected to other tests similar strong suspensions were used throughout, and were centrifuged in similar tubes. It follows that while with a 2% cell suspension in lactose solution it may be assumed that the concentration of the accurately prepared medium remains constant throughout, with a 10 or 15% cell suspension changes in cell volume will alter significantly the water content of the medium, and at the end of an experiment it was necessary to analyse both cells and medium. In addition, there were insufficient cells for duplicate tests. This introduces additional and significant sources of error.

*Chloride* was estimated by the wet-ashing method of Claudius (1924), because electrometric titrations, in a protein-rich haemolysate containing stromata, seemed to give a rather vague end-point.

*Experimental errors*

For cells in lactose solution with very low cation contents, the standard errors of the methods as judged from duplicate observations were as follows: Na ±2·1%, K ±1·6%, lactose ±2·3% and haemoglobin ±1·6%. The error in estimating $^{131}$I-albumin was rather high, because no scintillation counter was available, and with a liquid counter counts totalled about 2000, giving a counting error of ±2·2%, with an over-all error for duplicates of ±3·1%. For cation-rich cells in NaCl media, the S.E. for duplicate estimations (done on cells in narrow tubes) was for Na ±1·0% and for K ±0·7%. For chloride the error was ±1·4%. Other errors inherent in the various assumptions made are discussed later.

## RESULTS

This section is concerned with the composition of cells incubated in various media, and with the calculation of the osmotic coefficients.

### *Red cells incubated in lactose media*

#### *Composition of cells*

Results are shown in Table 1. The findings are similar to those recorded by Bolingbroke & Maizels (1960). In addition the Table shows the $^{131}$I-albumin space, that is, the percentage of the centrifuged cell column presumed to be occupied by extracellular $^{131}$I-albumin marker. The space is small for cells suspended in lactose solution for 1 hr, and increases with the duration of incubation; it is also much greater with cells suspended in 10% lactose medium than in 6% lactose medium. As a result, although the apparent volumes of cells shrunken and crenated in 6 and 10% lactose

TABLE 1. Changes in human red cells incubated in lactose media

| | Incubation time (hr at 37° C) | External lactose 0·186 osmolal, (6%, w/v) | | External lactose 0·311 osmolal, (10%, w/v) | |
|---|---|---|---|---|---|
| | | Mean ± S.D. | No. of observations | Mean ± S.D. | No. of observations |
| *V* | 0 | 1·11 | 2 | 0·86 | 3 |
| Cell pH at 20° C | 0 | 7·52 | 2 | 7·51 | 3 |
| Total lactose space (%) | 0 | 2·6 | 2 | 3·3 | 2 |
| $^{131}$I-albumin space (%) | 0 | 1·5 | 1 | 2·1 | 1 |
| Restricted lactose space (%) | 0 | 1·1 | 1 | 1·4 | 1 |
| Na (m-equiv/l. cells) | 0 | 9·0 | 2 | 8·1 | 3 |
| K (m-equiv/l. cells) | 0 | 75 | 2 | 102 | 3 |
| *V* | 1 | 0·73 | 2 | 0·71 | 3 |
| Cell pH at 20° C | 1 | 7·13 | 2 | 7·13 | 3 |
| Total lactose space (%) | 1 | 6·4 | 2 | 16·9 | 3 |
| $^{131}$I-albumin space (%) | 1 | 3·6 | 1 | 12·8 | 1 |
| Restricted lactose space (%) | 1 | 2·8 | 1 | 3·2 | 1 |
| Na (m-equiv/l. cells) | 1 | 4·4 | 1 | 3·8 | 3 |
| K (m-equiv/l. cells) | 1 | 24·1 | 2 | 14·9 | 3 |
| *V* | 3 | 0·71 ± 0·029 | 12 | 0·72 ± 0·028 | 10 |
| Cell pH at 20° C | 3 | 6·94 ± 0·07 | 12 | 6·88 ± 0·06 | 10 |
| Total lactose space (%) | 3 | 9·0 ± 1·4 | 12 | 23·7 ± 2·6 | 10 |
| $^{131}$I-albumin space (%) | 3 | 5·8 ± 1·0 | 6 | 18·8 ± 2·8 | 6 |
| Restricted lactose space (%) | 3 | 3·1 ± 0·15 | 6 | 4·1 ± 0·12 | 6 |
| Na (m-equiv/l. cells) | 3 | 2·6 ± 0·63 | 12 | 2·4 ± 0·54 | 10 |
| K (m-equiv/l. cells) | 3 | 12·7 ± 3·3 | 12 | 7·6 ± 2·2 | 10 |

Note: (i) *V* is the ratio of the cell volume to the volume of the fresh normal cell. (ii) The $^{131}$I-albumin marker is assumed to be extracellular, and the space it occupies is expressed as a percentage of the packed cell volume. The 'restricted lactose space' is obtained by deducting the $^{131}$I-albumin space from the *corresponding* total lactose space. As there are 10 or 12 observations on total lactose space and only 6 on the $^{131}$I-albumin space, only 6 of the former can correspond with the latter: hence the apparent discrepancy between the restricted lactose space and the difference between the total lactose space and the $^{131}$I-albumin space as recorded in the table.

solutions (estimated from the haemoglobin contents of standard dilutions of centrifuged cells), are after 3 hr roughly the same, about 71% of the normal cell volume, the true net volume of the cell in 6% lactose (at present regarded as the apparent volume minus the $^{131}$I-albumin space) is about 63%, while in 10% lactose solution it is only about 49% of the volume of the fresh normal cell.

Table 1 also shows that the lactose content of centrifuged red cells exceeds that which could be accommodated in the $^{131}$I-albumin space (excess lactose). This may be explained in one of three ways (Maizels & Remington, 1959*a*): (*a*) the excess lactose is extracellular and is distributed in a 'restricted' space available for small molecules, but not to such large molecules as $^{131}$I-albumin (or the Evans-blue–albumin complex); (*b*) the excess lactose is adsorbed at the cell surface; (*c*) the excess lactose has penetrated the cell. Clearly if the excess lactose is in or on the cell, then no restricted extracellular space exists at all. The matter is considered again later: for the present it is convenient to envisage a 'restricted lactose' space which increases slowly throughout incubation, and is rather higher with cells in 10% lactose solution than with those in the 6% medium (Table 1).

*Effects of lowering the lactose concentration of cell suspensions after a preliminary 3 hr period of incubation.* The true haemoglobin concentration of cells (as distinct from the haemoglobin concentration of the centrifuged cell mass) is about 14 m-mole/kg cell water for suspensions in 6% lactose medium and about 20 m-mole/kg for suspensions in 10% lactose medium, and the experiments so far described are incapable of giving data for the composition of cation-depleted cells whose haemoglobin is less than 13 m-mole /kg water. This defect cannot be remedied by incubating cells in media containing less than 6% lactose because haemolysis occurs, but it may be overcome by first depleting the cells of cation in 6% lactose solution and then adding distilled water after 3 hr; providing that mixing is thorough and rapid very little lysis now occurs, and cells may be obtained with only 5 m-mole haemoglobin/kg water.

If 1 vol. of a 3 hr suspension in 6% lactose solution is mixed with 3 or 4 vol. of water at room temperature, the external medium becomes acid (pH 6 approx.), whether because of $CO_2$ in the large volume of water added or from some other cause has not been determined, but the effect is to induce marked agglutination of the cells. This may be avoided by first adding enough dilute alkali to the water to bring the pH of the suspension to about 7. The effects of suspending red cells in very dilute lactose solution are shown in Table 2: it takes about 15 min to concentrate large volumes of cell suspension *before* the ultimate 25 min centrifuging is begun and for this reason initial records in the Table are designed '0–15 min'.

It will be seen that when the dilution of the lactose medium is marked, definite swelling of the cells occurs and may well be maximal for the particular lactose dilution used, because after the initial observation at '0–15 min', no further swelling occurs; indeed at '55–70 min' there is a slight but consistent shrinkage of the cells accompanied by loss of Na and K and possibly of other osmotically active substances.

TABLE 2. Composition of red cells incubated in grossly hypotonic lactose solution. The cells were incubated in 6 or 10 % lactose solution for 3 hr at 37° C; distilled water was then added and the suspension re-incubated

| | External lactose (osmolality after the addition of extra-water) | Time at 37° C (min) | Cells | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Cation (m-equiv/l.) | | $^{131}$I-albumin space (%) | Lactose space (%) | NaX + KX (osmolality) | Hb (m-mole/kg cell water) | Osmotic coefficient |
| No. | | | *V* | Na | K | | | | | |
| 1*a* | 0·186 | 0–15 | 0·70 | 2·37 | 11·0 | 5·4 | 7·9 | 0·056 | 15·1 | 8·7 |
| *b* | 0·093 | 0–15 | 0·80 | 1·65 | 7·8 | — | 6·6 | 0·031 | 11·4 | 5·4 |
| *c* | 0·093 | 55–70 | 0·78 | 1·35 | 6·5 | — | — | — | — | — |
| *d* | 0·0465 | 0–15 | 1·02 | 1·34 | 5·9 | 3·7 | 6·5 | 0·0225 | 7·8 | 3·1 |
| *e* | 0·0465 | 55–70 | 0·99 | 1·18 | 5·0 | — | — | — | — | — |
| *f* | 0·0372 | 0–15 | 1·16 | 1·25 | 5·1 | — | 7·8 | 0·019 | 6·7 | 2·7 |
| *g* | 0·0372 | 55–70 | 1·13 | 0·96 | 4·4 | — | — | — | — | — |
| 2*a* | 0·311 | 0–15 | 0·72 | 1·35 | 4·5 | — | 23·2 | 0·033 | 20·0 | 13·9 |
| *b* | 0·124 | 0–15 | 0·75 | 1·00 | 3·01 | — | 10·0 | 0·016 | 13·6 | 7·9 |
| *c* | 0·078 | 0–15 | 0·83 | 0·95 | 2·90 | — | 10·2 | 0·0145 | 11·4 | 5·6 |
| *d* | 0·052 | 0–15 | 0·97 | 0·75 | 2·47 | — | 8·2 | 0·0107 | 8·7 | 4·7 |

*V* = ratio of treated cell volume to normal cell volume. It is assumed that at the cell pH of 6·9, cell Na and K are not combined with haemoglobin, but form the highly dissociated salts NaX and KX. It is also assumed that the lactose in the centrifuged cell column is all extracellular. Water corresponding to the lactose space has been deducted from total water in the cell mass when calculating the osmotic coefficients.

It might be expected that as the cells swell in grossly hypotonic media the lactose space would diminish owing to obliteration of crenation, but in fact some crenation may be detected microscopically even in the swollen cells, and this probably accounts for a lactose space which is consistently greater than the lactose space of 3 % in centrifuged fresh normal cells (Table 2). It is probable that the almost complete removal of cation from the cells accounts for their ability to survive in a solution of lactose whose tonicity is only one eighth of the tonicity of the normal red cell.

*Calculation of osmotic coefficients*

This involves certain assumptions.

(*a*) The first is that the osmotic pressure of red cells and an external medium are the same. This is certainly true of normal red cells, for, as Sidel & Solomon (1957) and Paganelli & Solomon (1957) have shown, the exchange rate of water is several hundred thousand times faster than the exchange rate for cations, and though passive cation penetration may be

10–20 times faster than in normal cells, it would still be true to say that osmotic adjustments are virtually instantaneous, provided that the rate of water exchange is not decreased very greatly. This view is supported by Table 2, which shows that when water is added to a suspension of cells shrunken in lactose solution, cell swelling is probably complete at '0–15 min', and thereafter slight shrinking occurs, presumably from continued loss of cation. The possibility exists, however, that though capable of swelling in water, the greatly shrunken cell is incapable of further shrinking in a relatively hypertonic medium; if so, this, together with the relative or absolute impermeability to lactose, would prevent the adjustment of the osmotic level of the cell up to that of the external medium. Such an explanation could only apply to No. 2*a* of Table 2, because the cells of all the other suspensions already contain far more water than 2*a*, and could presumably adjust their osmotic pressures to that of the external medium by a further loss of water. Moreover, in spite of the low cation content of the lactose-treated cell, it is difficult to see how its osmotic pressure could have fallen below that of the external medium after 3 hr at 37° C, because the tonicities of the red cells and medium at the *start* of incubation were the same, and in these circumstances a relative decline in the osmotic pressure of the cells during incubation is not to be expected. In fact, the exact opposite occurs, because owing to the raised osmotic coefficient of haemoglobin in concentrated solution the osmotic pressure of the cells tends to rise, and isotonicity of cells and medium is only maintained by the passage from the cells of a grossly hypertonic effluent; this is discussed later.

(*b*) One of three assumptions may be made regarding the location of lactose in the column of centrifuged red cells (Maizels & Remington, 1959*a*). (i) The lactose may be extracellular, constituting a 'lactose space', part of this space being 'free' and corresponding to the $^{131}$I-albumin space, and part being 'restricted' and inaccessible to large molecules. If this is so, then both spaces (and especially the 'free space') increase during incubation in lactose solutions (Table 1), increase of the 'free space' presumably being due to gross crenation, and of the 'restricted space' to the development of minor irregularities in the cell surface. (ii) Part of the lactose corresponds to the $^{131}$I-albumin space, and the rest (extra lactose) is adsorbed at the cell surface: hence the lactose does not contribute to the osmotic pressure of the cells. There is no reason to suppose that this occurs, but the possibility cannot be excluded. (iii) The extra lactose has penetrated the cell; if so, an allowance must be made for its contribution to the osmotic pressure of the cell, while to obtain true cell water, water in the $^{131}$I-albumin space must be deducted from total water in the centrifuged mass. It may be noted that in the case of fresh cells in saline media con-

taining lactose as 'marker', no obvious penetration of lactose occurs, the lactose space being 3% of the packed cell volume at 0 hr and also at 1 hr; this conforms with Kozawa's (1914) view that lactose does not penetrate human red cells. Separate calculations have been made with each of the three assumptions mentioned, and these are represented in Fig. 2. Values for the osmotic coefficient in Table 2 and Fig. 1 are based on the assumption that all the lactose is extracellular.

(*c*) The pH of the cell haemolysates measured at 20° C, after cells had been incubated for 3 hr in lactose medium at 37° C, is about 6·9. It is assumed that the pH of haemolysate approximates to that of the interior of the unhaemolysed cell (see Methods), and that in these circumstances haemoglobin within the cells is not combined with ions.

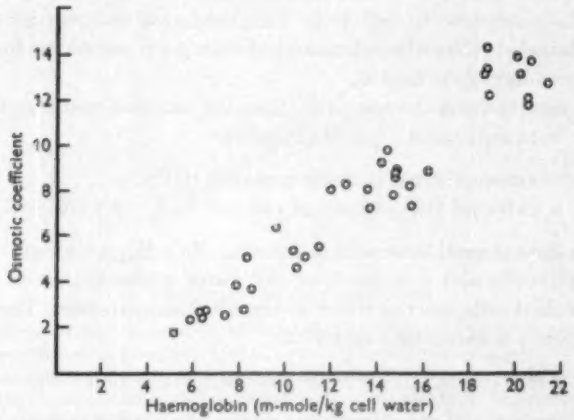



Fig. 1. Osmotic coefficients of haemoglobin in cells suspended in lactose solutions at 37° C, plotted against the corresponding m-molal concentrations of haemoglobin. It is assumed that all the lactose in centrifuged red cells is extracellular.

(*d*) It is assumed that the osmolality of Na and K salts within the cells is 1·9 times the molality of Na + K. This is correct for NaCl and KCl in a concentration of less than 0·02 m-molal, but if, as is possible, the cations are combined with phosphoric esters, the osmotic coefficient of the combined Na + K salts is virtually unknown. By analogy with inorganic phosphate this might be about 1·5 (Landolt-Bornstein's Tables, 1960), in which case the higher osmotic coefficients in Figs. 1 and 2 will be about 5% too low.

(*e*) It is assumed that the osmolality of lactose equals the molality; thus a solution of lactose 10 g/100 ml., containing 293 m-mole/l. solution or 311 m-mole/kg water, is assumed to have an osmolality of 0·311.

(*f*) Packed normal red cells after centrifuging for 30 min at 2000 *g* are assumed to contain 340 g haemoglobin and 700 g water/l. Alterations in cell volume and water content are calculated from changes in the haemoglobin content (see Methods).

(*g*) It is assumed that there is no significant liberation of osmotically active substances when red cells are incubated for 3 hr at 37° C. Clearly, any diffusible product of autolysis or any acidic product would not affect the calculation of the osmotic coefficient. It is true that Conway & McCormack (1953) have shown that with such highly organized tissues as diaphragm the freezing-point depression increases considerably when the tissue is macerated at room temperature. Nevertheless, when human red cells are incubated in a large volume of glucose-free electrolyte medium, increase in volume is usually less than 5% in 3 hr and is associated with a corresponding increase in cell base. This conforms with earlier results of Flynn & Maizels (1950) where human red cells were incubated for 18 hr in non-nutrient electrolyte media.

If these assumptions are accepted, then the osmotic coefficients may be calculated from equations (1) and (2) below:

$$\text{Apparent excess external osmotic pressure (OP)} = \text{external OP} - \text{apparent cell OP} = L_e - 1{\cdot}9\,(\text{Na}+\text{K})_i/w, \quad (1)$$

where $L_e$ is the external lactose in m-osmole, $(\text{Na}+\text{K})_i$ is the sum of Na + K in m-equiv/l. cells and $w$ is the true cell water content, that is, the total water in packed cells less the water in the total lactose space. The molality of haemoglobin is calculated as follows:

$$[\text{Hb}]\ (\text{mole/kg cell water}) = 340/(V \times w \times 66{,}700), \quad (2)$$

where $V$ is the ratio of the cell volume to the normal cell volume. Then the osmotic coefficient = (excess OP)/Hb molal. Data calculated in this way are shown in Fig. 1.

*Errors*

These fall into two groups: experimental errors, which are variable, and errors arising from the possible incorrectness of the assumptions given above.

*Summation of experimental errors.* For osmotic coefficients of 10 or more, errors in the values obtained for (Na + K) are of little significance, while errors in the estimation of haemoglobin alone may contribute an over-all error of ± 6%. In view of the number of terms in the relevant equations, it is not surprising that the summation of standard errors is about ± 11%. For less shrunken cells, with osmotic coefficients of 5 or less, errors in haemoglobin estimations are less significant, while errors in estimating

Na + K are more serious. In this group the S.E. is about ± 17 %. The scatter of points in Fig. 1 is an indication of the degree of experimental error.

*Errors due to unjustifiable assumptions.* It has been assumed that at the experimental pH (6·9 when measured at 20° C), all cell Na + K is combined with chloride, and that the osmolality of Na + K is 1·9 times the value of Na + K in m-equiv/l. cell water. If Na + K were in fact chiefly combined with phosphate, an error is introduced which has already been considered.

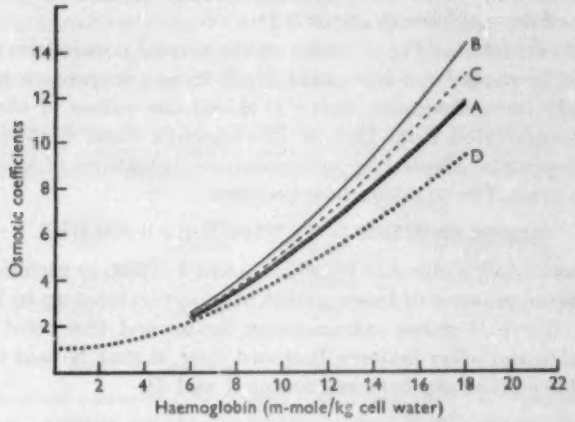



**Fig. 2.** Mean values of the osmotic coefficients of haemoglobin in red cells suspended in lactose solutions. *A* assumes that all lactose in centrifuged cells is extracellular; *B* assumes that lactose in excess of that present in the $^{131}$I-albumin space is adsorbed; *C* that the excess lactose is within the cell and contributes to its osmotic pressure. *D* is calculated from Dick & Lowenstein's (1958) virial equation (see text).

If, however, cell cation in lactose-treated cell were mainly combined with substances of low osmotic activity, including stroma proteins or other macromolecular structures, then the osmolality attributed to the cells would be too high, and the osmotic coefficients too low. Thus osmotic coefficients of about 12 would be 9 % too low and osmotic coefficients of about 3 would be 30 % too low. However this may be, errors arising from unjustifiable assumptions would not affect the general shape of the curve, and most of the conclusions reached would still be valid.

*Values for the osmotic coefficients of haemoglobin*

The molal osmotic coefficients obtained for haemoglobin in intracellular fluid containing 10–20 m-equiv Na + K/kg water are shown in Fig. 1. The data so summarized are derived from three sources: (*a*) cells incubated for

3 hr at 37° C in 10% lactose solution giving coefficients of about 12, (*b*) cells in 6% lactose solution giving coefficients of about 8, and (*c*) cells incubated for 3 hr in 10 or 6% lactose solutions followed by the addition of water. In this way it was possible to vary the volume of the cell and hence the concentration of haemoglobin, thus obtaining a whole range of osmotic coefficients from 1·8 upwards. It will be seen that there is a good deal of scatter, but it is clear that the osmotic coefficient rises progressively with increasing concentration of haemoglobin, and that in a system containing 18 m-mole haemoglobin/kg water (0·018 mole) the osmolality of haemoglobin is about 0·23.

Curves *A*, *B* and *C* of Fig. 2, based on the several possibilities of lactose distribution in packed red cells centrifuged from a suspension in lactose, have already been discussed; curve *D* shows the values of the osmotic coefficients calculated from Dick & Lowenstein's virial equation (1958), with the constants adapted to millimolal concentrations of haemoglobin and not to g/ml. The equation now becomes:

$$\text{osmotic coefficient} = 1 + 0{\cdot}106[\text{Hb}] + 0{\cdot}020[\text{Hb}]^2.$$

Dick & Lowenstein's curve is based on Adair's (1929) experimental data for the osmotic pressure of haemoglobin in concentrations up to 7 m-mole/kg water. Curve *D* shows extrapolation far beyond this point. Having regard to this and other matters discussed later, it may be said that there is reasonable conformity between curves *A* and *D*.

*Composition of the effluent from red cells suspended in lactose solution*

The tendency for the osmotic pressure of red cells in isotonic lactose solution to rise was noted by Bolingbroke & Maizels (1960), who based their views on the observation that the effluent from the cells in the process of shrinking had a higher tonicity than that of the original cells or of the medium in which they had been suspended. Their figures, however, were too high because they took no account of the fact that trapped intercellular water increases as cells shrink, so that the actual loss of water is greater than is suggested by the simple decrease in the apparent volume of the cells, as judged by changes in the haemoglobin content of the centrifuged cell column. If now calculations are based on the more complete data set out in Table 1, it may be shown that in 3 hr cells lose 90 m-equiv Na + K/l. original cells ($0{\cdot}86 \times 110 - 0{\cdot}72 \times 10{\cdot}0$) together with 282 ml. water (140 ml. by manifest shrinking and 142 ml. by shift of water from within the cells to the extracellular space). Hence the effluent contains 90 m-equiv Na + K in 282 ml. water or 312 m-equiv Na + K/l. water, compared with 168 m-equiv Na + K/l. cell water at 0 hr. Its osmolality is thus considerably in excess of that of the original cells, and also of the medium in which they

were suspended. Thus the value, though less than Bolingbroke & Maizels' original figure, is still very high, and the finding can only mean that as the red cell shrinks in isotonic lactose solution its osmolality tends to rise, and equilibrium is maintained by the discharge of a hypertonic effluent.

*Red cells in hypotonic, isotonic and hypertonic sodium chloride solutions*

*Changes in the cells*

In these experiments suspensions of red cells (about 10 %) were prepared in solutions containing 105, 155, 350 and 450 mM-NaCl. As the pH of suspensions varied between 6·8 and 6·95, the distribution of water between cells and medium varied slightly. Moreover, albumin added as buffer contained variable traces of NaCl. Hence the Na concentration of any

TABLE 3. Observations on human red cells thrice washed in isotonic NaCl solution, then suspended in NaCl solutions of varying tonicities containing 2 % serum albumin (w/v), and centrifuged immediately at 2000 *g*. pH of the suspending medium 6·9; temp. 20° C

| External medium Na + K (m-equiv/kg water) | | Red cells | | | |
|---|---|---|---|---|---|
| | *V* | Lactose space (%) | Na + K (m-equiv/kg water) | Haemoglobin (m-mole/kg water) | Osmotic coefficient |
| 110 | 1·31 | 3·0 ± 0·3 | 108 | 5·3 | 2·4 |
| 159 | 1·03 | 3·4 ± 0·3 | 153 | 7·3 | 3·3 |
| 340 | 0·70 | 4·3 ± 0·4 | 286 | 13·4 | 8·5 |
| 430 | 0·65 | 5·6 ± 0·6 | 330 | 15·9 | 12·0 |

*V* = ratio of cell volume to the volume of fresh normal cells. For measuring the lactose space, lactose 1 g/100 ml. was added to the external medium and the concentration of NaCl reduced accordingly. Cell Na + K and haemoglobin have been corrected for intercellular water. Lactose space measured in 6 expts. Other data from 10 expts.

particular medium varied slightly from one experiment to another. For this reason only the average findings of 10 experiments are shown in Table 3, except in the case of data concerning the intercellular space. It will be seen that as the external cation concentration is raised the intercellular space increases moderately, the excess of external Na + K concentration over the cell cation concentration also increases, and the osmotic coefficient rises sharply.

*Calculation of osmotic coefficients*

The assumptions are similar to those made earlier, but the calculation is less satisfactory, because while in lactose-treated cells the absolute amount of anion and cation is very small, the anion and cation contents of cells in NaCl media are high, and in the thrice-washed cells about 18 % of cation is combined with phosphoric esters; this requires further consideration. From Farmer & Maizels' (1939) data it appears that at pH 6·9, 1 atom P in cell phosphoric esters binds 1·8 m-equiv cation. Such dissociation constants of

these esters as are available suggest that the osmotic coefficients of their salts will be similar to those of inorganic phosphate at the same pH. But there seems to be no information about the osmotic coefficients of inorganic phosphate in the presence of high concentrations of chloride, nor indeed is it clear how the true concentration of phosphate may be assessed in the presence of high concentrations of haemoglobin. For NaCl and KCl in 0·1 molal solution an osmotic coefficient of 1·85 will suffice and for 0·4 molal, 1·78. For similar solutions at pH 6·9 containing 20% of cation combined with phosphate it is suggested that this amount of cation multiplied by 1·5 will give the osmolality of phosphate salts in NaCl solutions of lower cation concentrations, while a factor of 1·4 may be used for high concentrations (see phosphate data in Landolt & Börnstein's tables, 1960). Since the chloride content was estimated in all these cases, osmolality may be calculated as follows: Suppose cells contain 150 m-equiv Na + K and 120 m-equiv Cl/kg water, 120 m-equiv Na + K will be combined with Cl, and it is assumed that the residual 30 m-equiv Na + K are mainly combined with phosphate; hence the osmolality is taken as $(120 \times 1{\cdot}84 + 30 \times 1{\cdot}5)/1000$ or 0·266. Osmotic coefficients are calculated as before and equal the excess of external over apparent cell osmolality divided by the haemoglobin in m-mole/kg cell water. Values for cell constituents are corrected for the presence of trapped intercellular medium, as measured with 1% lactose added as indicator substance.

*Errors*

Errors arising from the preceding are not formidable. Thus if the extreme assumptions be made that the phosphate factor of 1·4 has an absolute error of $\pm 0{\cdot}15$ and that the equating of phosphate-bound cation with [Na + K]–[Cl] has an error of $\pm 20\%$, then it may be shown that the maximum error from this source is $\pm 1$ for osmotic coefficients of 9 and $\pm 0{\cdot}9$ for coefficients of 12. It is in fact unlikely that errors would be as great as this or that they would be additive. A more serious error arises from the fact that in this group of experiments calculation of the osmotic coefficients involves a figure which is the difference of two relatively large values. Thus in the example given above, where the osmolality of the cells was 0·266, the external osmolality would be about 0·286, and experimental errors might contribute $\pm 0{\cdot}0045$ to the difference of 0·020 calculated, corresponding in this case to a coefficient of $3 \pm 0{\cdot}7$. In the case of cells in hypertonic NaCl with an apparent osmolal difference of 0·2 this source of error is much less significant, affecting osmotic coefficients of about 10 by $\pm 0{\cdot}5$. In fact, the probable over-all error at this level is about 0·9, i.e. $10 \pm 0{\cdot}9$; for coefficients of 2 or 3, the error is also about $\pm 1$.

*Osmotic coefficients*

These are summarized in Table 3. The values for the osmotic coefficients there are calculated from the mean values shown. In order to obtain the S.D. it is necessary to plot the data of each experiment and read off the osmotic coefficients corresponding to 5, 7, 13 and 16 m-mole haemoglobin/kg cell water. The means and S.D. of these may then be calculated. For Hb 0·005 molal, the osmotic coefficient was $2{\cdot}2 \pm 1{\cdot}0$; for Hb 0·007 molal,

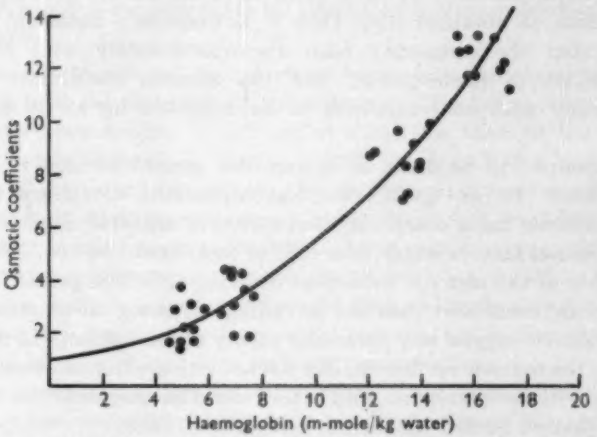



Fig. 3. Osmotic coefficients of haemoglobin in cells suspended in NaCl solutions at 20° C. Data have been corrected for the extracellular space as measured with lactose. The curve shows mean values.

$3{\cdot}2 \pm 1{\cdot}0$; for Hb 0·013 molal, $8{\cdot}2 \pm 1{\cdot}2$; for Hb 0·016 molal, $12{\cdot}0 \pm 1{\cdot}1$. In view of all the assumptions made, it will be clear that these figures are quite approximate, but they suggest that the osmotic coefficients of haemoglobin in cells in NaCl solutions with a high concentration of cell electrolyte do not differ greatly from the osmotic coefficients of haemoglobin in cells suspended in lactose media, where the cell concentration of cation is low.

## DISCUSSION

Figures 1 and 2 and data in the preceding section show that the osmotic coefficients of haemoglobin rise progressively with increasing concentration of haemoglobin, the data conforming with Dick & Lowenstein's (1958) virial equation based on Adair's (1929) figures. When it is recalled that Adair used pure haemoglobin solutions at 0° C, while the present experiments were conducted at 37 or 20° C with intact cells containing stroma and

lipids equal to one-tenth the weight of haemoglobin (Maizels & Paterson, 1937), it is not surprising that there is some divergence between the respective curves. Moreover, the virial equation has been based on Adair's data for haemoglobin concentrations between 0 and 7 m-mole/l. solution, while curve *D* (Fig. 2) has been extrapolated to 18 m-mole/kg water. With this in mind, and also the relatively high error of the present methods, it may be said that for concentrations between 5 and 18 m-mole/kg water satisfactory agreement exists between the curve for osmotic coefficients obtained experimentally by the present methods, and the extrapolation of Adair's data as obtained from Dick & Lowenstein's equation. It thus appears that the osmolality rises disproportionately with increasing concentration of haemoglobin, and the osmotic coefficients increase continuously with concentrations of haemoglobin up to 20 m-mole/kg water.

No attempt will be made to explain the present findings in terms of 'bound water', for, as Ogston (1956) has emphasized, the concept that each solute molecule has a sharply limited sphere of influence implies a knowledge of causal factors which does not, in fact, exist. So too, Dick (1959) in a review of the osmotic behaviour of living cells, has pointed out that the 'osmotic coefficient' has the advantage of being an empirical term, which does not suggest any particular theory of mechanism. In many real solutions the osmotic coefficient, like the activity coefficient, shows marked variation with concentration, and in the case of haemoglobin this is evident in the equation quoted by Dick & Lowenstein (1958):

$$\phi = 1 + Ax + Bx^2,$$

where $\phi$ is the osmotic coefficient of haemoglobin, $x$ its concentration, and $A$ and $B$ are arbitrary constants.

The anomalous rise in the osmotic pressure of proteins with concentration is attributed to the marked increase in the entropy of mixing which arises from disproportion between the small molecules of the solvent and the large molecules of solute. The subject is discussed by Dick (1959), who also gives references, and will not be considered further here. It is, however, pertinent to consider the state of haemoglobin within cells. Perutz (1948) calculates that in the normal red cell with a haemoglobin content of 34 g/100 ml. cells (7·3 m-mole/kg water), the average distance between molecules is 75 Å. He suggests that this would just permit free rotation of cylindrical molecules whose effective dimensions are 63 Å diameter and 40 Å height (including a monomolecular layer of 'bound water' 3 Å thick). More recently Perutz, Rossman, Cullen, Muirhead, Will & North (1960) have described the haemoglobin molecule as a spheroid 64 Å long, 55 Å wide and 50 Å high. This would require 64 + 6 or 70 Å for free rotation,

corresponding to 9 m-mole/kg water. It follows that the continuous rise in the osmotic coefficient is associated with an increase in the concentration of haemoglobin molecules, whether these are free to rotate at concentrations below 9 m-mole/kg water, or whether they suffer increasing restriction of movement at concentrations above. In fact, with concentrations of 20 m-mole haemoglobin/kg water, where the osmotic coefficient is still rising (Fig. 1), the average distance between molecules is only 64 Å and molecular movements must be very restricted indeed.

*Biological effects of the increase in the osmotic coefficients of haemoglobin with concentration*

Maizels (1935) invoked bound water to explain the observation that when red cells are shrunken in hypertonic NaCl solution, the apparent electrolyte concentration in cell water is far less than in the external medium, while Roepke & Baldes (1942) attributed the high vapour pressure of shrunken red cells to the anomalous osmotic coefficient of haemoglobin in high concentration, basing this view on Adair's (1929) data. Again, it has long been known that red cells in hypotonic solution swell less and in hypertonic solution shrink less than would be expected from simple osmotic considerations, and Dick & Lowenstein (1958) have shown that this finding may be ascribed to variation in the osmotic coefficient of haemoglobin at high and low concentrations.

In the anaemias the haemoglobin content of red cells may fall to 20 g/100 ml. or less, water increasing correspondingly. Since the plasma salt concentration is about normal, cell cation must rise to effect a balance and in severe cases this may be as high as 14 % (Maizels, 1936). Moreover, with a decline in haemoglobin concentration from 7·5 to 3·7 m-mole/kg water, osmolality from this source alone falls by about 0·016 (6 % of the total osmolality) and it must be assumed that the increase in cell cation is required to cover not only the rise in cell water, but also the fall in the osmolality of haemoglobin. In congenital haemolytic icterus, on the other hand, the reverse occurs (Maizels, 1936): haemoglobin may rise to 40 g/100 ml. and water fall to 65 g/100 ml. with a rise in the osmolality of haemoglobin of 0·013, and it is likely that the fall in water content and rise in the osmolality of haemoglobin are together responsible for a fall in cation content per litre of cells, which may exceed 10 %.

Finally, a rising osmotic coefficient with increasing concentration is characteristic of all proteins, but so far as the present writers know, the pathological implications have not yet been explored. Clearly, in conditions of salt depletion such as may occur from vomiting or excessive sweating fall in the osmotic coefficient of cell protein will limit to some extent the amount of water that must enter the cells to effect compensation.

So too, in association with a cerebral lesion (Allott, 1939), or with the dehydration of infantile diarrhoea, a rise of plasma Na occurs which may reach 200 m-equiv/l. water; here a rise in the osmotic coefficient of the cell proteins could by itself account for 7% of the total osmolality of the cells, and in this way limit withdrawal of water from the tissues.

SUMMARY

(1) Red cells in a solution of non-electrolyte like lactose lose anion, cation and water, and shrink. They also become highly crenated so that the intercellular space among centrifuged cells increases, the increase varying with the duration of suspension and the concentration of the external medium. Thus in 6% lactose solution the lactose space averages 6·4% at 1 hr and 9·0% at 3 hr; in 10% lactose the value at 3 hr is 23·7%. The $^{131}$I-albumin space is 3–4% less than the lactose space and also increases with time. In view of the extent of the intercellular space (which is only 2–3% in packed normal cells), an allowance must be made for it when calculating the concentration of substances in the water of cells incubated in lactose media.

(2) When red cells have been incubated in lactose media, the osmolality of the external lactose exceeds the osmolality of Na and K salts within the cells. If it be assumed that the osmotic balance is maintained by haemoglobin, then since the concentration of haemoglobin is known, its osmotic coefficient may be calculated. If the external concentration of lactose is varied, cell volume and hence the haemoglobin concentration alter: in this way data may be obtained for the osmotic coefficients of haemoglobin. When haemoglobin is 7 m-molal, the osmotic coefficient is about 3, and when haemoglobin is 20 m-molal the osmotic coefficient is about 12 and its osmolality 0·24.

(3) As a result, when red cells shrink in lactose solution from loss of electrolyte and water, the osmotic pressure of the cells tends to rise, and osmotic equilibrium is maintained by the discharge from the cells of an effluent whose osmolality is about twice that of the original cells and of the lactose medium in which they were suspended.

(4) The concentration of haemoglobin in red cells has also been altered by suspending the cells in varying concentrations of sodium chloride. In these cells, which are relatively rich in electrolyte, the osmotic coefficients of haemoglobin do not differ greatly from the osmotic coefficients of haemoglobin in the electrolyte-poor cells of lactose suspensions, though the data suggest that they are perhaps somewhat higher.

## REFERENCES


ADAIR, G. S. (1929). Thermodynamic analysis of the observed osmotic pressures of protein salts in solutions of finite concentration. *Proc. Roy. Soc.* A, **126**, 16–24.

ALLOTT, E. N. (1939). Sodium and chlorine retention without renal disease. *Lancet*, **236**, 1035–1037.

BOLINGBROKE, V. & MAIZELS, M. (1959). Calcium ions and the permeability of human erythrocytes. *J. Physiol.* **149**, 563–585.

CLAUDIUS, M. (1924). A new method for the micro-determination of chlorine in blood. *Acta med. scand.* **61**, 4–7.

CONWAY, E. J. & MCCORMACK, J. I. (1953). The total intracellular concentration of mammalian tissues compared with that of the extracellular fluid. *J. Physiol.* **120**, 1–14.

DICK, D. A. T. (1959). Osmotic properties of living cells. *Int. Rev. Cytol.* **8**, 387–448.

DICK, D. A. T. & LOWENSTEIN, L. M. (1958). Osmotic equilibria in human erythrocytes studied by immersion refractometry. *Proc. Roy. Soc.* B, **148**, 241–256.

FARMER, S. N. & MAIZELS, M. (1939). Organic anions of human erythrocytes. *Biochem. J.* **33**, 280–289.

FLYNN, F. V. & MAIZELS, M. (1949). Cation control in human erythrocytes. *J. Physiol.* **110**, 301–318.

GOLD, C. L. & SOLOMON, A. K. (1954). The transport of sodium into human erythrocytes *in vivo*. *J. gen. Physiol.* **38**, 389–404.

HARRIS, E. J. & MAIZELS, M. (1952). Distribution of ions in suspensions of human erythrocytes. *J. Physiol.* **118**, 40–53.

HENDERSON, L. J. (1928). *Blood. A Study in General Physiology*. New Haven: Yale University Press.

KOZAWA, S. (1914). Beiträge zum arteigenen Verhalten der roten Blutkörperchen. III. Art-differenzen in der Durchlässigkeit der roten Blutkörperchen. *Biochem. Z.* **60**, 231–256.

LANDOLT-BÖRNSTEIN (1960). *Zahlenwerte und Funktionen aus Physik, Chemie, Astronomie, Geophysik und Technik*, 6th ed. Berlin: Springer-Verlag.

MAIZELS, M. (1935). The permeation of erythrocytes by cations. *Biochem. J.* **29**, 1970–1982.

MAIZELS, M. (1936). The anion and cation contents of normal and anaemic bloods. *Biochem. J.* **30**, 821–830.

MAIZELS, M. & PATERSON, J. L. H. (1937). Base binding in erythrocytes. *Biochem. J.* **31**, 1642–1656.

MAIZELS, M. & REMINGTON, M. (1959*a*). Percentage of intercellular medium in human erythrocytes centrifuged from albumin and other media. *J. Physiol.* **145**, 658–666.

MAIZELS, M. & REMINGTON, M. (1959*b*). Cation exchanges of human erythrocytes. *J. Physiol.* **145**, 641–657.

OGSTON, A. G. (1956). Remarks on the definition and measurement of the solvation of macromolecules in solution. *Proc. Int. Wool Textile Res. Conf.* (1955). Vol. B, 92–97.

PAGANELLI, C. V. & SOLOMON, A. K. (1957). The rate of exchange of tritiated water across the human red cell membrane. *J. gen. Physiol.* **41**, 259–277.

PERUTZ, M. F. (1948). Submicroscopic structure of the red cell. *Nature, Lond.*, **161** (i) 204–205.

PERUTZ, M. F., ROSSMAN, M. G., CULLIS, A. F., MUIRHEAD, H., WILL, G. & NORTH, A. C. T. (1960). Structure of haemoglobin. *Nature, Lond.*, **185**, (i), 416–422.

ROEPKE, R. R. & BALDES, E. J. (1942). A study of the osmotic properties of erythrocytes. *J. cell. comp. Physiol.* **20**, 71–93.

SIDEL, V. W. & SOLOMON, A. K. (1957). Entrance of water into human red cells under an osmotic pressure gradient. *J. gen. Physiol.* **41**, 243–257.

*J. Physiol.* (1961), **155**, *pp.* 46–58
*With 6 text-figures*
*Printed in Great Britain*

# CHANGES IN POTASSIUM CONCENTRATION AROUND MOTOR NERVE TERMINALS, PRODUCED BY CURRENT FLOW, AND THEIR EFFECTS ON NEUROMUSCULAR TRANSMISSION

BY A. TAKEUCHI* AND N. TAKEUCHI

*From the Department of Physiology, University of Utah College of Medicine, Salt Lake City, Utah, U.S.A.*

(*Received* 12 *July* 1960)

Since the discovery of the spontaneous miniature end-plate potentials (Fatt & Katz, 1952), many conditions which alter their frequency have been found (temperature: Fatt & Katz, 1952; Boyd & Martin, 1956; Liley, 1956*a*; Takeuchi, 1958; osmotic pressure: Fatt & Katz, 1952; Furshpan, 1956; stretch: Hutter & Trautwein, 1956; tetanus: Liley, 1956*b*; Brooks, 1956; Hubbard, 1959; electrotonus: del Castillo & Katz, 1954*b*; Liley, 1956*c*; potassium ion concentration: Liley, 1956*c*; drugs: Brooks, 1956; Kraatz & Trautwein, 1957; Furukawa, Furukawa & Takagi, 1957). These changes in frequency are believed to be due to the alteration of conditions at motor nerve terminals.

At present recording methods do not permit direct measurement of electrical changes at motor nerve terminals. In the experiments to be described the frequency of miniature end-plate potentials was used as an index of changes in the motor nerve terminals produced by several experimental procedures. These were designed to alter the potassium concentration around the nerve terminals and to detect the resultant changes in miniature frequency and neuromuscular transmission. For this purpose inward current was passed through the muscle membrane at the end-plate region. The evidence to be presented indicates that such current reduces the potassium concentration around the nerve terminals and in high-potassium medium produces significant changes in miniature frequency and in transmission. The related problem of the post-tetanic increase in miniature frequency has also been investigated.

## METHODS

Experiments were performed on the sartorius muscle of summer frog (*Rana pipiens*) at room temperature (20–23° C). The sartorius muscle was mounted in a lucite (methacrylate resin) chamber with a volume of about 4 ml. Bathing solutions were changed by running at

* Fellow of the Rockefeller Foundation.

least 20 ml. of fresh solution through the chamber. The muscle was left for at least 10 min in each solution before measurement.

Normal Ringer's solution was used of the following composition (mM): NaCl 115; KCl 2·0; $CaCl_2$ 2·0, with added phosphate buffer to keep pH near 7·0. In addition, prostigmine bromide ($1 \times 10^{-6}$ g/ml.) was used to increase the amplitude of miniature end-plate potentials (m.e.p.p.s). In order to increase the concentration of potassium, $K_2SO_4$ was substituted for NaCl. In some cases the frequency of miniature discharge was increased by raising the osmotic pressure of bathing fluid by adding NaCl or sucrose to normal Ringer's solution (Fatt & Katz, 1952), or by replacing sodium ions by ammonium ions (Furukawa *et al.* 1957).

Micro-electrodes filled with 3 M-KCl were used for potential and current electrodes. The focus of an end-plate was located under microscopical controls by recording m.e.p.p.s of largest amplitude and steepest time course and superficial fibres were used for experiments. The current electrode was connected to a square-pulse generator through a 100 MΩ resistor in series and was inserted within 100 $\mu$ of the recording electrode. Electrical changes were recorded by a cathode-ray oscilloscope through a cathode follower pre-amplifier and d.c. amplifier. The membrane potential was measured from the cancelling potential which brought the potential beam back to the original position on the cathode-ray tube. In some cases end-plate current (e.p.c.) was recorded by use of the voltage clamp technique (Takeuchi & Takeuchi, 1959).

## RESULTS

### *Effect of current on the spontaneous m.e.p.p.*

When the potassium concentration in the bathing solution was increased, the frequency of spontaneous m.e.p.p.s was increased and usually several minutes elapsed before the change in frequency developed fully. When the preparation was returned from high-potassium solution to one of low potassium, the frequency remained rather high for some time and took about 30 min or more to return to its original value. The frequency of miniature discharge in normal Ringer's solution and the rate of change in frequency when the potassium concentration was altered were different in each end-plate. Relative frequencies in various potassium concentrations as compared to those in normal solution are presented in Fig. 1. Each symbol indicates the mean frequency recorded from an individual end-plate. A marked change in frequency was obtained with increase in potassium concentration to greater than about 5 mM, but smaller changes were observed in lower concentrations. This result is similar to that reported by Liley (1956*c*) with the mammalian end-plate.

After the frequency of miniature discharge became stationary in high-potassium solution, an inward current was passed through muscle membrane by the current electrode, hyperpolarizing the membrane. The current influenced both the amplitude and the frequency of the m.e.p.p. As shown in Fig. 2, when the muscle membrane was hyperpolarized the amplitude of miniature discharge was increased and its frequency decreased. After cessation of the current the amplitude and the frequency returned to their original value. The mean amplitude of the m.e.p.p. had an almost linear relation to the change of membrane potential, as was

observed with the end-plate potential (e.p.p.; Fatt & Katz, 1951). When an inward current was suddenly passed through muscle membrane, the amplitude of the miniature discharge increased rapidly as the membrane was hyperpolarized, but the decrease in frequency developed gradually, becoming nearly constant only about 5–10 sec after the start of current.

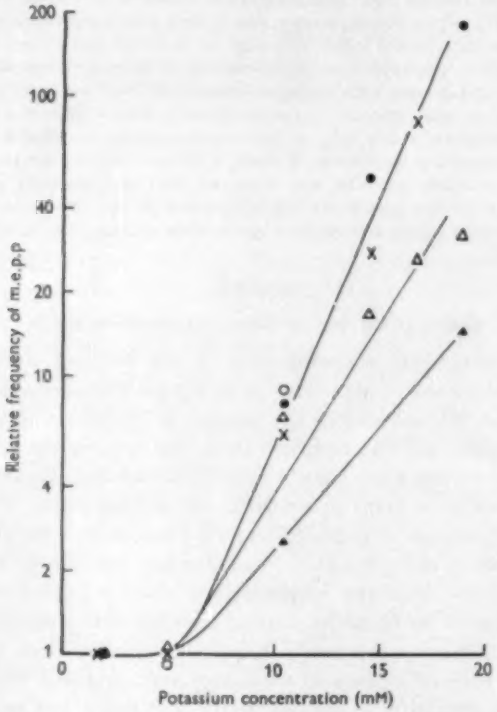



Fig. 1. Relative frequencies in various potassium concentrations as compared to those in normal solution. Each sign indicates mean frequency obtained from an individual end-plate. Semi-log. scale.

After the termination of current the frequency gradually increased and returned to its original value after about 10 sec. In Fig. 3 two examples recorded in 12 mM-$K^+$ are shown, each point representing mean frequency of miniature discharge for each 400 msec. Beginning at the downward arrows, inward current was passed, which hyperpolarized the membrane by 33 and 38 mV in *A* and *B* respectively. After the start of current about 0·5 sec elapsed before the membrane potential reached a steady hyperpolarized level. During this period the miniature frequency could not be

measured. Following this, however, the frequency change was gradual and reached a fairly steady level after 5–10 sec. At the upward arrows the current was terminated and the frequency gradually increased, returning to near its original value after about 10 sec.

In Fig. 4*A* the mean frequency of the miniature discharge recorded from the same end-plate in various media is plotted against the increase in membrane potential produced by inward current. Filled circles represent the mean frequencies obtained in 10·4 mM-$K^+$ and crosses and open circles

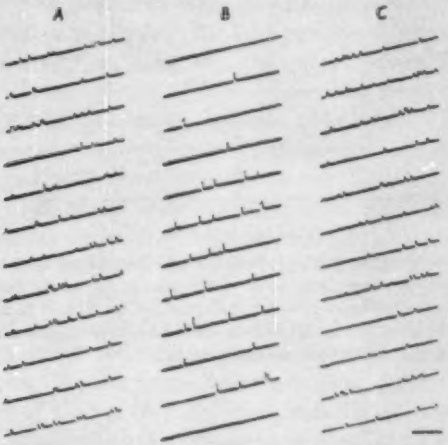



Fig. 2. Effect of current on m.e.p.p. in high-potassium medium (10·4 mM). *A*, before passing current. *B*, while passing current, hyperpolarizing the membrane by 59 mV. *C*, after cessation of current. Time marker, 50 msec.

are those obtained in 14·6 and 16·7 mM-$K^+$ respectively. The relation between frequency and membrane hyperpolarization was almost linear, with possibly a slight upward concavity.

Several possible explanations of the change in the frequency of the miniature e.p.p. may be considered. (1) A part of the current through the muscle membrane passed through the nerve ending, changing its membrane potential. (2) The current through the muscle membrane altered the sensitivity of the end-plate membrane or interfered with the diffusion of transmitter. (3) The ionic composition of the fluid around the nerve endings was changed by passing current through the muscle membrane. The linear relationship between m.e.p.p. amplitude and membrane potential suggests that the current does not interfere significantly with the sensitivity of the end-plate. The possibility that the electric current disturbs the diffusion of transmitter through the space between nerve terminals and end-plate membrane may also be excluded. Since the

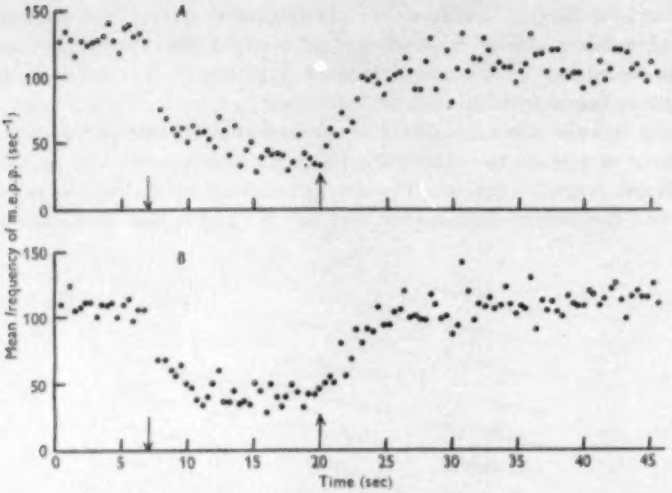



*Fig. 3. Time course of the change in miniature discharge frequency by current flow.* Each point represents mean frequency of 400 msec in 12 mM-$K^+$ Ringer's solution. Between arrows the membrane was hyperpolarized by 33 and 38 mV in *A* and *B* respectively. For about 0·5 sec after the start of current frequency was not measured because of instability of the membrane potential.

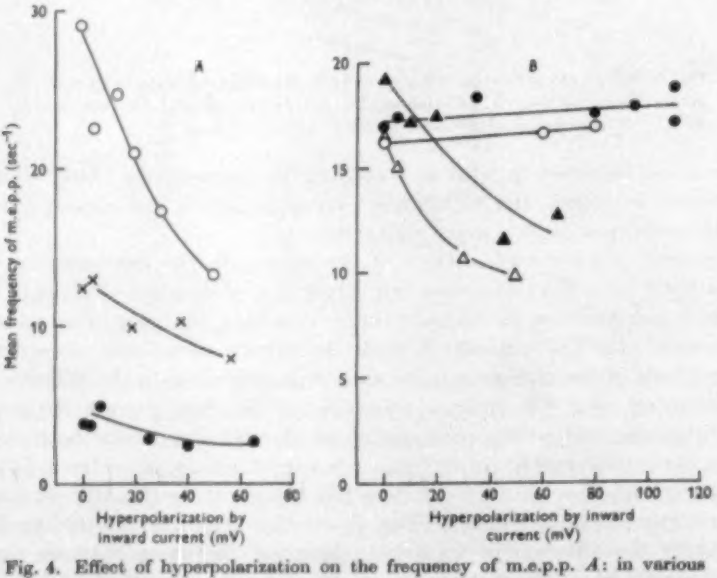



Fig. 4. Effect of hyperpolarization on the frequency of m.e.p.p. *A*: in various potassium concentrations, ○, 16·7 mM; ×, 14·6 mM; ●, 10·4 mM. *B*, ○●, 1·5 times hypertonic solution; △▲, 18·8 mM-$K^+$.

direction of the current is inward through the muscle membrane and acetylcholine (ACh) is positively charged, the current would not impede the diffusion of transmitter but rather accelerate it.

If the inward current through the muscle membrane were to pass through the nerve ending its direction through the nerve terminal membrane is most likely to be outward. This would be expected to depolarize the nerve terminals and increase the frequency of the m.e.p.p., contradicting the present results. Liley (1956*c*) observed that after the onset of electrotonic polarization of the nerve terminals the frequency attained its final value almost instantaneously. In the present experiments, however, about 10 sec was necessary for the frequency change to develop fully after the start of the current. Furthermore, the frequency change was manifest only in high-potassium medium while when the discharge frequency was increased in hypertonic solution or in ammonium solution, inward current had little or no influence on the frequency of the m.e.p.p.s (cf. Fig. 4*B*). These results exclude the first explanation.

The most probable explanation appears to be that the muscle membrane has a relatively low permeability to sodium ion and that inward current is carried mainly by inward-moving potassium ion and outward-moving chloride ion. The potassium ion concentration in the space between nerve terminals and muscle membrane would be reduced by the flow of inward current, decreasing the discharge frequency of m.e.p.p. If this were the case the amount of change in concentration could be estimated from the frequency change in various potassium concentrations. As shown in Fig. 4*A*, in 16·7 mM-$K^+$ the current which hyperpolarized the membrane by about 50 mV decreased the frequency from 29 to 13·2/sec. The latter value corresponds to the frequency obtained from same end-plate in 14·6 mM-$K^+$. Thus in this case it may be supposed that the current decreased the potassium concentration near muscle fibre by about 2 mM. In normal solution inward current had little influence on the discharge frequency. This may be due to the fact that in these circumstances the concentration changes of $K^+$ that might occur on passing inward current would be expected to have little influence on the discharge frequency (cf. Fig. 1).

The effect of outward current on the discharge frequency was not investigated extensively, because when the membrane was depolarized the amplitude of the miniature discharge became small and measurement of frequency was difficult.

*Effect of current on the neuromuscular transmission*

The change in discharge frequency of m.e.p.p.s by the passage of inward current appears to result from a change in potassium concentration around

the nerve terminals. In this section the effect of such current on neuromuscular transmission in high-potassium medium was investigated. The amplitude of the e.p.c. clamped at a hyperpolarized potential by a short pulse of about 15 msec was compared with that clamped at same potential by a long pulse (longer than 10 sec). As is shown in Table 1*A*, in a high-potassium medium the amplitude of the e.p.c. clamped at hyperpolarized

TABLE 1. Comparison of amplitude of e.p.c. hyperpolarized by short (15 msec) and long pulses (longer than 10 sec)

| End-plate | Amplitude of e.p.c. hyperpolarized by: Short pulse ($\times 10^{7}$ A) | Long pulse ($\times 10^{7}$ A) | Hyperpolarization (mV) | Ratio of amplitudes (%) | $K^{+}$ (mM) | $Mg^{2+}$ (mM) |
|---|---|---|---|---|---|---|
| | | | *A.* High-potassium solution | | | |
| *a* | 1·40 | 1·15 | 66 | 82 | 18·8 | 10 |
| *b* | 2·52 | 2·06 | 66 | 82 | 6·0 | 10 |
| *b* | 2·39 | 1·81 | 66 | 76 | 12·0 | 10 |
| *c* | 2·18 | 1·93 | 54 | 88·5 | 6·0 | 10 |
| *c* | 2·40 | 1·97 | 54 | 81 | 6·0 | 10 |
| *d* | 1·40 | 1·17 | 53 | 82·5 | 4·0 | 3·5 |
| *e* | 3·04 | 2·68 | 37 | 88·5 | 7·0 | 0 |
| *e* | 3·25 | 2·81 | 48 | 86·5 | 7·0 | 0 |
| | | | *B.* Normal solution | | | |
| *f* | 1·45 | 1·46 | 70 | 101 | 2·0 | 0 |
| *g* | 3·20 | 3·40 | 39 | 106 | 2·0 | 0 |
| *h* | 0·87 | 1·07 | 65 | 123 | 2·0 | 0 |
| *i* | 1·21 | 1·22 | 61 | 100 | 2·0 | 0 |
| *j* | 0·78 | 0·76 | 53 | 97·5 | 2·0 | 0 |

TABLE 2. Effect of potassium concentration on mean quantum content ($m$) of e.p.p. in Ringer's solution containing (mM) Mg 25, Ca 0·8

| End-plate | 1·5 mM-K | 10 mM-K | 1·5 mM-K | 10 mM-K |
|---|---|---|---|---|
| *a* | 0·76 | 1·32 | 0·74 | — |
| *b* | 0·32 | 0·45 | 0·17 | 0·38 |

Quantum content ($m$) was calculated from the relation $m = \log_e$ (number of impulses/number of failures) (del Castillo & Katz, 1954*a*). Each value was calculated from 160 to 200 impulses.

potentials by short pulses was larger than that clamped with long pulses, although no striking difference could be detected in normal solution (Table 1*B*).

In order to clarify the reason for this result, the quantum content was measured with an end-plate in high magnesium and low calcium, following which the potassium concentration was increased with the same concentration of magnesium and calcium. Table 2 shows that in high-potassium medium the quantum content increased. The result in the preceding section suggested that the change in potassium concentration took place rather slowly after the hyperpolarization of membrane. Thus the difference in

amplitude of e.p.c. with the membrane hyperpolarized by short or by long pulses may be interpreted as follows: when the membrane was hyperpolarized by short pulses there may have been little change in potassium concentration near the muscle membrane, and the quantum content of e.p.c. was rather large, but when the membrane was hyperpolarized with long pulses a change in potassium concentration may have occurred and

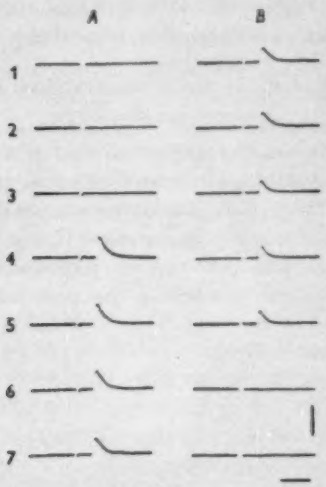



**Fig. 5.** E.p.c. evoked every 3 sec in 12 mM-$K^+$ solution. Between *A* 2 and *A* 3 membrane was suddenly hyperpolarized (61 mV), and e.p.c. appeared in *A* 4. *B* 1 and *B* 2 were recorded during hyperpolarization; between *B* 2 and *B* 3 membrane potential was returned to resting value and neuromuscular transmission was blocked after *B* 6. Current scale, $2 \times 10^{-7}$ A; time scale, 5 msec.

the quantum content may have decreased. It is known that KCl relieves the curare block at the neuromuscular junction (Wilson & Wright, 1937; Brown & von Euler, 1938; Walker & Laporte, 1947) and the increase in quantum content in high-potassium solution may be one of the reasons for the decurarizing action of potassium ion.

In some cases, when 7·5 mM-$K^+$ Ringer's solution was introduced neuromuscular transmission was blocked and no potential change could be obtained by applying supermaximal stimuli to the nerve. In such instances hyperpolarization of muscle membrane restored neuromuscular transmission in a few seconds. Figure 5 shows e.p.c. evoked every 3 sec. Between *A* 2 and *A* 3 the membrane potential was suddenly hyperpolarized by 61 mV. The e.p.c. appeared in *A* 4 and thereafter transmission continued. *B* 1 and *B* 2 are e.p.c.s obtained during the hyperpolarization and between *B* 2 and *B* 3 the membrane was returned to its resting potential.

Transmission continued for about 10 sec after the cessation of inward current and then was blocked suddenly. The time course of the recovery of transmission by current flow was similar to that of the change in frequency of m.e.p.p. in high potassium medium. This sudden block of transmission may occur around the arborizations of nerve fibres, suggesting that the change in concentration of potassium ion was not confined to the nerve terminals or the groove between nerve terminal and the end-plate membrane, but also occurred in a wider area around the nerve arborization.

### *Effect of current on the post-tetanic increase in frequency of miniature discharge*

Various investigators have hypothesized that post-tetanic potentiation can be explained in terms of an increased external potassium ion concentration (cf. Hughes, 1958). Recent observations have shown that after a tetanus frequency of the m.e.p.p. is increased (Liley, 1956*b*; Brooks,1956; Hubbard, 1959). Since this post-tetanic potentiation of discharge frequency runs a time course paralleling the post-tetanic potentiation of

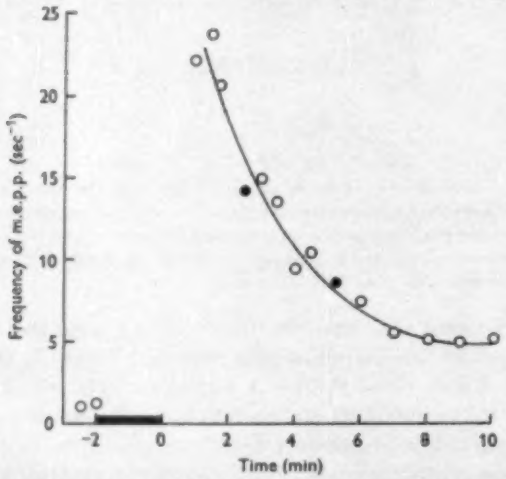



Fig. 6. Effect of current on post-tetanic increase of miniature discharge frequency. Each circle represents mean frequency for about 10 sec. ○, without current; ●, during passing inward current, hyperpolarizing the membrane about 50 mV. During thick bar tetanic stimulus (10/sec) was applied.

response, there is good reason for postulating a common mechanism. The increase in miniature discharge frequency might also be explained in terms of an increased external potassium ion concentration. This hypothesis was tested by passing inward current through the muscle membrane during post-tetanic potentiation of discharge frequency. If the increase in dis-

charge frequency were due to an increased potassium concentration around nerve terminals, inward current would be expected to reduce the potassium concentration near the muscle membrane and the discharge frequency might decrease during and after passing current. In Fig. 6 the changes in mean frequency of m.e.p.p.s in such an experiment are shown. During the thick bar a 10/sec tetanus was applied. After the tetanus the miniature frequency increased to more than 20 times its control value and then decayed gradually. Open circles show mean frequencies obtained without current, and the filled circles are those obtained when the membrane was hyperpolarized by about 50 mV. The result indicates that inward current did not change the time course of the recovery of miniature discharge frequency. This result is contrary to the above hypothesis, i.e. that the post-tetanic potentiation of miniature discharge is due to the increased potassium concentration around nerve terminals.

## DISCUSSION

The present results suggest that the passage of electric current through the muscle produces an appreciable change in concentration of potassium ion near the muscle membrane, at least at the end-plate. Since, if ion mobilities were uniform as in free solution, concentration changes would not occur by passing current, the change near the muscle may be due to a selective permeability of the resting muscle membrane. The resting muscle is permeable to potassium and chloride ions, and inward current through the membrane may consist mainly of inward-moving potassium ions and outward-moving chloride ions. If mobilities and diffusion constants of ions are uniform everywhere, other than in the membrane, potassium ions carried through the membrane will be replaced by ions brought to the membrane by diffusion and by current. If the stretch of end-plate is assumed to be small compared with the characteristic length of muscle fibre, the current density may be considered as uniform throughout the end-plate region. If the end-plate is assumed to be on the exposed surface of the muscle fibre, the change in concentration near the end-plate can be represented approximately as a one-dimensional diffusion problem. The potassium concentration $C$ at time $t$ and at distance $x$ normal to the membrane is a solution of the following equation:

$$D\frac{\partial^2 C}{\partial x^2} = \frac{\partial C}{\partial t},$$

$$C = C_0 \quad (t = 0),$$

$$D\frac{\partial C}{\partial x} = \frac{V g_K}{F} - p \quad (x = 0),$$

$$C \to \infty \quad (x = \infty).$$

The amount of potassium ion carried by current through unit area of membrane in unit time is $Vg_K/F$, where $V$ is potential change produced by current through muscle membrane (outside potential minus inside potential). $F$ is the Faraday constant, and $g_K$ the potassium conductance per unit area of membrane and $D$ the diffusion constant. $p$ is the amount of potassium ion carried by current to a unit area of membrane per second and a function of the concentration and the potential gradient in solution. In the present conditions this amount may be negligibly small compared to $Vg_K/F$, because the potassium concentration in solution is much less than that of other ions. $g_K$ is a function of potassium concentration and it is assumed that $g_K = aC$, where $a$ is a constant. When the muscle membrane potential is near the potassium equilibrium potential, $g_K$ is approximately

$$P_K \frac{F^2 V_m}{(RT)^2} K_o \left(1 - \frac{K_o}{K_i}\right)^{-1},$$

where $V_m$ is membrane potential (outside potential minus inside potential) and $K_o$ and $K_i$ are outside and inside concentrations of potassium ion respectively. If $V_m$ and $P_K$ are assumed to be constant and $K_o$ is much smaller than $K_i$, $g_K$ may be considered directly proportional to $C$. The solution of the diffusion equation with these initial and boundary conditions can be easily obtained by Laplace transformation (cf. Crank, 1956), and

$$C = C_0 \left[ \operatorname{erf}\left(\frac{x}{2\sqrt{Dt}}\right) + e^{hx+Dth^2} \left\{1 - \operatorname{erf}\left(\frac{x}{2\sqrt{Dt}} + h\sqrt{Dt}\right)\right\}\right].$$

At $x = 0$ the solution is reduced to

$$C = C_0 e^{Dth^2}\{1 - \operatorname{erf}(h\sqrt{Dt})\},$$

where

$$\operatorname{erf}(Z) = \frac{2}{\sqrt{\pi}} \int_0^Z e^{-u^2} du \quad \text{and} \quad h = \frac{Va}{DF} \quad (p \text{ is neglected}).$$

If the current which changes the membrane potential by 50 mV in 16·7 mM-$K^+$ changes the potassium concentration near the muscle membrane by 2 mM in 10 sec and $g_K$ is assumed to be about 500 $\mu$mho/cm$^2$ at $C$ = 10 mM (cf. Hodgkin & Horowicz, 1959), the diffusion constant should be about $4{\cdot}5 \times 10^{-7}$ cm$^2$/sec. This value is much smaller than that of KCl in aqueous solution (in the order of $1{\cdot}6 \times 10^{-5}$ cm$^2$/sec). The calculation is merely an approximation but suggests that some structures might act as diffusion barriers.

The fact that the inward current had no influence on the post-tetanic increase in discharge frequency seems to exclude the possibility that the post-tetanic frequency change is due to an increase in potassium concentration around nerve terminals. Recent observation shows that post-

tetanic potentiation of synaptic transmission can be explained by an increase in the membrane potential and amplitude of action potential of presynaptic fibres (Lloyd, 1949; Eccles & Krnjević, 1959). However, it seems difficult to explain the post-tetanic increase of miniature discharge in terms of increased membrane potential and its mechanism remains obscure.

SUMMARY

1. The effect of inward current through the muscle membrane on the potassium concentration near muscle membrane was investigated by using the frequency of miniature end-plate potentials (m.e.p.p.) as an index of potassium concentration.

2. The frequency of spontaneous m.e.p.p. was markedly increased in concentrations of potassium greater than about 5 mM, smaller changes being observed in lower concentrations.

3. The frequency of miniature discharge in high-potassium medium was decreased by passing inward current through the muscle membrane at end-plate region. After the cessation of current the frequency returned to original level.

4. The change in frequency of miniature discharge after the start and the cessation of inward current was rather slow and 5–10 sec elapsed before the frequency reached fairly stationary level.

5. These results can be explained by a decrease in potassium concentration around nerve terminals during passage of inward current.

6. Inward current relieved the block of neuromuscular transmission occurring in high-potassium medium. The amplitude of end-plate current during hyperpolarization by short pulses was larger than that during long pulses. These results can also be explained in terms of changes in potassium concentration.

7. Inward current had no influence on the post-tetanic increase of miniature discharge frequency, which excludes the possibility that the post-tetanic increase of miniature discharge frequency is caused by increase in external potassium concentration around nerve terminals.

The authors wish to express their thanks to Drs C. C. Hunt and A. R. Martin for their advice and criticism.

REFERENCES

Boyd, I. A. & Martin, A. R. (1956). Spontaneous subthreshold activity at mammalian neuromuscular junctions. *J. Physiol.* **132**, 61–73.

Brooks, V. B. (1956). An intracellular study of the action of repetitive nerve volleys and of botulinum toxin on miniature end-plate potentials. *J. Physiol.* **134**, 264–277.

Brown, G. L. & von Euler, U. S. (1938). The after effects of a tetanus on mammalian muscle. *J. Physiol.* **93**, 39–60.

Crank, J. (1956). *The Mathematics of Diffusion*. Oxford: Clarendon Press.

DEL CASTILLO, J. & KATZ, B. (1954*a*). Quantal components of the end-plate potential. *J. Physiol.* **124**, 560–573.

DEL CASTILLO, J. & KATZ, B. (1954*b*). Changes in end-plate activity produced by presynaptic polarization. *J. Physiol.* **124**, 586–604.

ECCLES, J. C. & KRNJEVIĆ, K. (1959). Presynaptic changes associated with post-tetanic potentiation in the spinal cord. *J. Physiol.* **149**, 274–287.

FATT, P. & KATZ, B. (1951). An analysis of the end-plate potential recorded with an intracellular electrode. *J. Physiol.* **115**, 320–370.

FATT, P. & KATZ, B. (1952). Spontaneous subthreshold activity at motor nerve endings. *J. Physiol.* **117**, 109–128.

FURSHPAN, E. J. (1956). The effects of osmotic pressure changes on the spontaneous activity at motor nerve endings. *J. Physiol.* **134**, 689–697.

FURUKAWA, T., FURUKAWA, A. & TAKAGI, T. (1957). Fibrillation of muscle fibers produced by ammonium ions and its relation to the spontaneous activity at the neuromuscular junction. *Jap. J. Physiol.* **7**, 252–263.

HODGKIN, A. L. & HOROWICZ, P. (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol.* **148**, 127–160.

HUBBARD, J. I. (1959). Post-activation changes at the mammalian neuro-muscular junction. *Nature, Lond.*, **184**, 1945–1947.

HUGHES, J. R. (1958). Post-tetanic potentiation. *Physiol. Rev.* **38**, 91–113.

HUTTER, O. F. & TRAUTWEIN, W. (1956). Neuromuscular facilitation by stretch of motor nerve-endings. *J. Physiol.* **133**, 610–625.

KRAATZ, H. G. & TRAUTWEIN, W. (1957). Die Wirkung von 2, 4-Dinitrophenol (DNP) auf die neuromuskuläre Erregungsübertragung. *Arch. exp. Path. Pharmak.* **231**, 419–439.

LILEY, A. W. (1956*a*). An investigation of spontaneous activity at the neuromuscular junction of the rat. *J. Physiol.* **132**, 650–666.

LILEY, A. W. (1956*b*). The quantal components of the mammalian end-plate potential. *J. Physiol.* **133**, 571–587.

LILEY, A. W. (1956*c*). The effects of presynaptic polarization on the spontaneous activity at the mammalian neuromuscular junction. *J. Physiol.* **134**, 427–443.

LLOYD, D. P. C. (1949). Post-tetanic potentiation of response in monosynaptic reflex pathways of the spinal cord. *J. gen. Physiol.* **33**, 147–170.

TAKEUCHI, A. & TAKEUCHI, N. (1959). Active phase of frog's end-plate potential. *J. Neurophysiol.* **22**, 395–411.

TAKEUCHI, N. (1958). The effect of temperature on the neuromuscular junction of the frog. *Jap. J. Physiol.* **8**, 391–404.

WALKER, S. M. & LAPORTE, Y. (1947). Effects of potassium on the end-plate potential and neuromuscular transmission in the curarized semitendinosus of the frog. *J. Neurophysiol.* **10**, 79–85.

WILSON, A. T. & WRIGHT, S. (1937). Anti-curare action of potassium and other substances. *Quart. J. exp. Physiol.* **26**, 127–139.

*J. Physiol.* (1961), **155**, *pp.* 59–71
*With 0 text-figures*
*Printed in Great Britain*



# THE EFFECT OF CERTAIN ANAESTHETICS ON THE ACTIVITY OF SMALL MOTOR FIBRES SERVING THE HIND LIMB OF THE RAT

By B. L. ANDREW

*From the Department of Physiology, Queen's College, University of St Andrews, Dundee*

(*Received* 18 *July* 1960)

In their recent study of the activity in small motor fibres in the nerve supply of the gastrocnemius muscle of the decerebrate rabbit, Diete-Spiff & Pascoe (1959) noted that certain volatile anaesthetics, namely, ether, ethyl chloride, chloroform and trichlorethylene had a strong stimulatory effect, whereas chloralose, hexobarbitone sodium, and thiopentone sodium depressed the discharge. In their experiments urethane was found to act as a stimulant both in the decerebrate animal and in intact animals already anaesthetized with urethane. It seemed of interest to investigate further the relation between these effects and the simultaneously produced changes in reflex excitability in alpha and gamma fibres.

In the past nearly all the work on the mammalian small motor fibres has been carried out on the cat, and a number of reflex responses, both spinal and supraspinal have been described in the decerebrate, spinal or lightly anaesthetized animal (Hunt, 1951; Granit, Job & Kaada, 1952; Eldred & Hagbarth, 1954; Hunt & Paintal, 1958). In the present experiments many of the procedures which have been used to elicit small motor fibre responses in the cat have been tried on the lightly anaesthetized rat, and certain of these which have been found to produce reflex effects with regularity have been selected and used to follow the changes which occur when anaesthetics are given to the lightly anaesthetized animal. Preliminary reports of part of this work have already appeared (Andrew, 1959, 1960).

## METHODS

The experiments were performed on twenty-six adult rats. Anaesthesia was induced with trichlorethylene vapour and maintained by intraperitoneal injection of urethane solution, 25 g/100 ml., 4 ml./kg body weight. Further injections were given as necessary. In four experiments, pentobarbitone sodium, and in two other experiments thiopentone sodium were used instead of urethane as the basal anaesthetic. The level of anaesthesia was controlled so that it was deep at the beginning of the experiment during dissection and was permitted to lighten later when nerve activity was examined. In some of the animals flexion

withdrawal reflexes could be aroused in the hind limb by stimulation of the toes, but there was no stretch reflex activity in the hind limb extensor muscles.

*Dissection.* Nerve dissection was carried out under liquid paraffin. A binocular dissecting microscope was used. As the rat's leg is rather small, there was usually not enough skin available to form the raised edges of a pool of liquid paraffin over the nerve and muscles to be dissected; it was found convenient to fit an oiltight silver-foil boundary wall to the wound cavity with a depth of 1 cm. The limb was not denervated, and in order to disturb the muscle innervation as little as possible recordings were made usually from the central ends of small intramuscular nerve filaments. Where recordings were made from one of the larger trunks, e.g. the peroneal nerve, the epineurium was removed with scissors without interrupting the nerve. The perineurium was then slit longitudinally with needle points and slender nerve filaments picked off the main bundles. In some preparations, in order to loosen the bundles of nerve fibres, a small volume of hyaluronidase solution (about 100 i.u. dissolved in a fraction of a millilitre of Locke's solution) was run into the opened nerve trunk.

*Recording.* The electrodes were bright silver wires, and were connected to conventional capacity-coupled amplifiers and thence to cathode-ray oscillographs and loudspeaker. The action potentials were recorded on moving bromide paper.

*Inhalation.* The head of the rat was inserted into the open end of a Perspex box 7 cm long and 4 × 4 cm cross-section. A tube entered the box opposite the mouth of the animal. In experiments with nitrous oxide and oxygen mixtures, and bromochlorotrifluoroethane (Fluothane; Imperial Chemical Industries) and air mixtures, the gas mixture was passed into the box and escaped with expired gases through the space between the animal's neck and the mouth of the box. To produce anoxia the air in the box was diluted with nitrogen in the same way. The responses to ether, ethyl chloride, chloroform and trichlorethylene were obtained by placing cotton-wool soaked in the anaesthetic in the box and partly withdrawing the animal's head. Tracheal cannulae were not used, and on the few occasions that respiratory obstruction developed the animal was discarded.

*Nomenclature.* In the literature, the efferent fibres which have been proved or presumed to operate the intrafusal muscle fibres have been referred to as gamma efferents, small motor fibres, fusimotor fibres, spindle motor fibres and intrafusal motor fibres. In this paper the terms gamma efferents or small motor fibres will be used. The vasomotor efferents, although detectable in many of the nerve filaments used in this work, are not included in this category.

## RESULTS

Of the basal anaesthetics that have been tried, urethane has proved to be the most reliable; there is a large margin of safety with increasing dosage, it does not depress respiration unduly and the anaesthetic lightens slowly and in a predictable way throughout an experiment lasting several hours. Thiopentone sodium, pentobarbitone sodium and chloralose were given trials but found less satisfactory. The following description of reflex activity in gamma efferents refers therefore primarily to the rat lightly anaesthetized with urethane; the results with thiopentone sodium and pentobarbitone sodium as the basal anaesthetic were qualitatively similar. No useful results were obtained with chloralose.

A group of reflexes has been selected to study the action of anaesthetics given in addition to the basal anaesthetic. They are all easily elicited by natural forms of stimulation (that is, they do not involve electrical stimula-

tion of nerve trunks) and they are differentially sensitive to additional anaesthetic given either by inhalation or injection. Table 1 gives a summary of these reflexes and includes for completeness the reflex response to anoxia and certain inhaled anaesthetics.

TABLE 1. Reflexes operating on gamma efferents used in the present study

| | Afferent stimulus | Recording site | Effect |
|---|---|---|---|
| (*a*) Spinal | Toe pinch, ipsilateral | Flexor muscle nerves | Excitation |
| | | Gastrocnemius nerve | Inhibition |
| | Toe pinch, contralateral | Gastrocnemius nerve | Excitation |
| | Heel pinch, ipsilateral | Gastrocnemius nerve | Excitation |
| (*b*) Supra-spinal | Touch in ext. auditory meatus (head-shake reflex) | Limb muscle nerves | Phasic excitation |
| | Pinna pinch | Limb muscle nerves | Prolonged excitation |
| | Inhaled ether, ethyl chloride, chloroform, trichlorethylene | Limb muscle nerves | Excitation |
| | Anoxia | Limb muscle nerves | Excitation |

In the description of the results it has been assumed that, in general, the relative sizes of action potentials in records of the activity of a group of nerve fibres are related to the nerve-fibre diameters; the validity of this assumption in the case of efferents to skeletal muscle has been examined and supported by Hunt (1951). In the rat, when electrodes are placed on the central end of a nerve trunk supplying one of the larger limb muscles, it is usually not possible to detect the impulses in the gamma fibres. These become detectable when the nerve is split into very slender filaments. In this situation there is usually little difficulty in distinguishing, on the basis of action-potential size, between alpha and gamma fibre action potentials. When, however, records are made of efferent activity in small intramuscular nerve branches, the results suggest that there is less difference in fibre diameters. This is conjecture, but in any case it is convenient to retain the terms alpha and gamma, even though in this recording position they may not be accurate descriptions of fibre diameter. In these situations the main clues to identification are found in the temporal pattern of action-potential activity.

*Toe-pinch reflexes.* The receptive field extends over all five toes and the adjacent skin of the sole of the foot. The pinch was applied manually with blunt forceps. The excitation of the gamma fibres to the flexor muscles and the inhibition of the active gamma fibres to the gastrocnemius muscle on the ipsilateral side is assumed to be part of the flexion withdrawal reflex, and it occurs irrespective of whether the anaesthetic level prevents the alpha motoneurones from discharging. The response of a tonically discharging gamma fibre of a flexor muscle and the beginning of the discharge in an alpha fibre are shown in Fig. 1. The excitation of gamma

fibres of the contralateral limb appeared to affect both flexor and extensor muscles.

*Heel-pinch reflex.* The receptive field extends over the heel of the ipsilateral foot and includes the hairy skin over the Achilles tendon. There is a quite sharp demarcation line between the heel zone of the sole, which causes excitation of the gastrocnemius gamma efferents, and the toe zone, which causes inhibition of the same fibres. Exploration with a probe while recording from the peripheral end of the sural nerve showed that both zones are within the innervation field of this nerve. In some animals very few of the gastrocnemius gamma fibres maintained a tonic discharge; however, their presence in a nerve filament could be revealed by starting a short discharge by pinching the heel region. The beginning of a discharge in two gamma fibres to the lateral gastrocnemius muscle in response to heel-pinch can be seen in Fig. 2(*a*).

*Head-shake reflex.* If a probe is moved gently in the opening of the external auditory meatus, the animal shakes its head after a short latency, presumably in an attempt to dislodge the foreign body. During the oscillatory movements of the head a short volley of impulses is discharged in gamma fibres to hind-limb muscles. In some instances larger fibres become active also. Presumably this discharge forms part of the postural compensation of the limbs during the shaking movements of the head. An example is given in Fig. 3.

*Pinna-pinch reflex.* Pinching the pinna with forceps produces, after a brief interval, a general excitation of limb gamma fibres which usually extends beyond the duration of the stimulus. In some animals alpha fibres were also stimulated and slight movements of the limbs occurred. The pinch may be enough to start a tonic discharge in a hitherto inactive fibre. The receptive zone is confined to the pinna itself, pinching the surrounding skin is ineffective. An example is given in Fig. 4(*a*).

*Anoxia.* The stimulant effect of anoxia on gamma fibres is a general one and is exerted supraspinally. It is usually delayed until anoxia is severe and corresponds in time to the massive discharge in the sympathetic fibres and protrusion of the eyeballs. The supraspinal nature of this excitation has been shown in the cat by Schulte, Busch & Henatsch (1959).

*Resting discharge.* There appeared to be a large variation between different animals in the number of tonically discharging gamma fibres. As a general rule it seemed that the lighter the anaesthesia, the larger the number of active fibres, the more labile the discharge rate, and the greater the reflex accessibility of the gamma motoneurones to sensory stimulation. The discharge rate in some preparations underwent spontaneous changes of frequency, a cyclical rise and fall of frequency with a periodicity of a

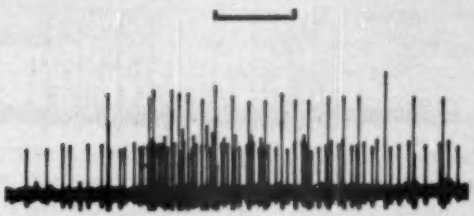


Fig. 1. The responses to ipsilateral toe pinch in group of fibres serving a hind-limb flexor muscle. The fibre with the small action potential which discharged throughout the record was tonically excited when ethyl chloride was inhaled by the animal. The fibre with the largest action potential, which was presumed to be an alpha fibre, was not caused to discharge tonically by ethyl chloride inhalation and gave reduced response to toe pinch after several minutes of inhalation and after the disappearance of head-shake and pinna-pinch responses. Time marker, 1/5th sec.

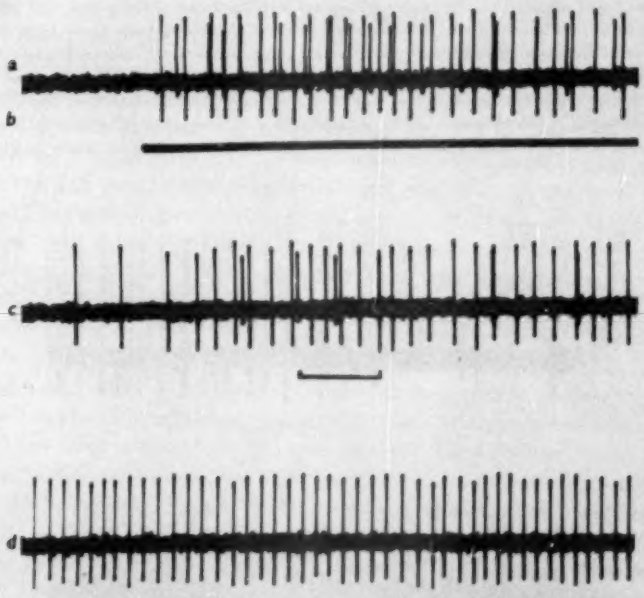



Fig. 2. Records (*a*), (*c*) and (*d*) are from the same gamma fibres dissected from the nerve to the lateral gastrocnemius muscle. The signal marker (*b*) indicates pinch applied to the heel of the foot and applies to the three records. (*a*) shows the control excitation. (*c*) shows the much reduced response which modulates the continuous discharge after 45 sec of inhalation of ethyl chloride. (*d*) shows the unresponsiveness of the tonic discharge to the same stimulus after 2¼ min inhalation. Time marker, 1/5th sec.

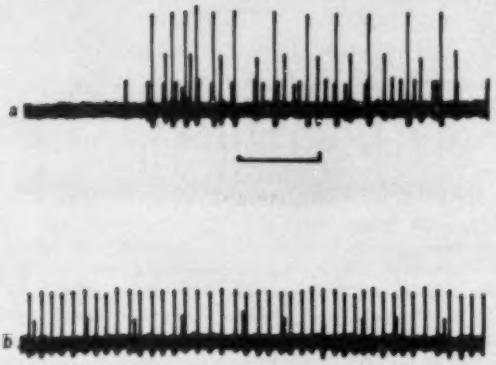



Fig. 3. Records (*a*) and (*b*) are from the same filament dissected from the central end of the peroneal nerve. (*a*) shows the short phasic excitation which occurred in three fibres during the oscillatory movements of the head-shake reflex. (*b*) After 2½ min of inhalation of trichlorethylene vapour the intermediate sized fibre had begun to discharge continuously and at about this time the head-shake reflex failed to influence the discharge in all three fibres. The largest fibre could still be activated by the flexion withdrawal reflex, but there was no phase of tonic excitation due to the anaesthetic: it was presumed to be an alpha fibre. The smallest fibre was tonically excited but to a lesser extent than the intermediate sized fibre. Time marker, 1/5th sec.

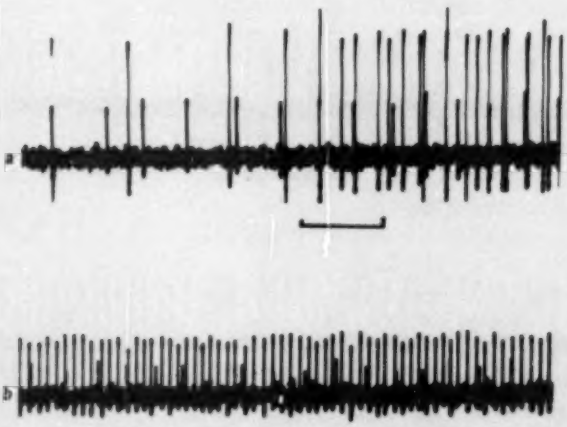



Fig. 4. Records (*a*) and (*b*) are from the same filament dissected from the central end of the peroneal nerve. (*a*) shows the increasing activity as the pinna was pinched, both fibres were accelerated. (*b*) Shows the activity after 2 min of ethyl chloride inhalation. The smaller fibre seen in record (*a*) was now discharging continuously at 50 impulses/sec and another smaller fibre had been recruited. Time marker, 1/5th sec.

fraction of a minute was occasionally seen. In these rather active preparations, a loud sound would sometimes cause a great increase in activity and the recruitment of hitherto inactive gamma fibres.

*The effects of additional anaesthetic*

*Urethane.* In four experiments the effects of a series of doses of urethane on the reflex and tonic activity of alpha and gamma fibres were examined. The injections were intravenous in two and intraperitoneal in the others and were continued until respiration stopped. The dose required to produce respiratory arrest in an animal that has been anaesthetized for some hours is relatively large and in these experiments was 370, 625, 650 and 875 mg. The temporal order of effects as the dosage of urethane was increased was as follows: resting discharge stopped, the supraspinal reflexes, i.e. head-shake, ear-pinch and the response to ethyl chloride disappeared, alpha-fibre component of flexor reflex disappeared, and finally the gamma response to toe-pinch (ipsilateral). At no stage was any excitation of gamma fibres detected. In one of these animals there was a slight reflex movement of the muscles operating on the pinna when the latter was pinched; this movement persisted after the excitatory linkage to the limb gamma motoneurones had been interrupted.

*Inhalation anaesthetics, type 1: ethyl chloride, ether, trichlorethylene and chloroform.* The characteristic feature of this group was that they produced a phase of progressive excitation during which the frequency of the tonic discharge rose to a maximum. The response occurred with each of the three basal anaesthetics used, namely urethane, pentobarbitone sodium and thiopentone sodium. A description will be given first of a typical response to trichlorethylene. Usually four phases could be recognized. During the first half minute of inhalation there were signs of arousal such as movements of the vibrissae and changes in respiratory rhythm; at this time the gammas accelerated slightly and tonic discharges in alpha fibres sometimes began. This phase was absent in some animals. Then followed 3 or 4 min during which alpha activity stopped and resting discharges, if any, in gamma fibres ceased. At about the fourth or fifth minute, the phase of excitation in gamma fibres began, and a quite regular tonic discharge of rising frequency occupied the next few minutes. The discharge then ceased abruptly or continued for a short time as a series of high-frequency trains of impulses and then ceased. By this time the animal was deeply anaesthetized; the head-shake and pinna-pinch reflexes disappeared during the phase of excitation. The extinction of the heel-pinch reflex before the end of the phase of tonic excitation of the gamma fibres is shown in Fig. 2. The toe-pinch response in nerves to flexor muscles was rather more resistant, and some modulation of the discharge could be heard at the end of the

excitation phase. During the ensuing few minutes after the rat was returned to breathing air the gammas might pass back through the excitation phase and the reflexes returned in the reversed sequence. The maximum frequencies achieved during the phase of excitation were variable, and the range extended from a change from zero–28 impulses/sec to zero–110 impulses/sec; that is, within the normal limits of frequencies observed when gamma fibres are activated reflexly. In recordings from multi-fibre preparations it was seen that the phase of excitation did not

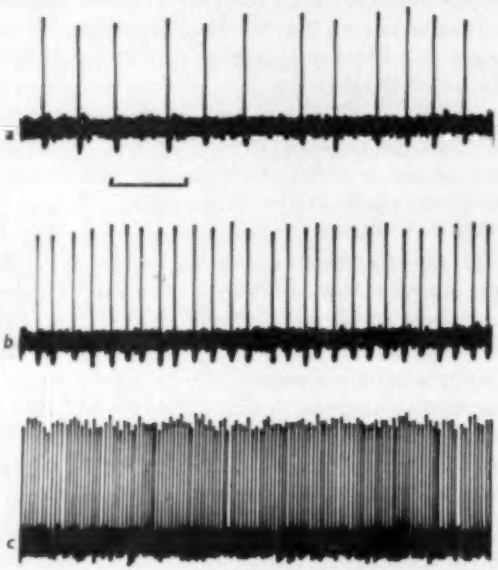



Fig. 5. Response to the inhalation of ethyl chloride vapour in a single gamma efferent in an intramuscular nerve branch serving the medial gastrocnemius muscle. Control record (*a*) shows the resting discharge, 10 impulses/sec; (*b*) after 60 sec inhalation, discharge rate 22 impulses/sec; (*c*) after 80 sec inhalation, discharge rate 105 impulses/sec. Time marker, 1/5th sec.

begin synchronously in all fibres and towards the end of the phase some became quiescent before others. The response to ethyl chloride was more rapid, the tonic excitatory phase began in 1 min or less but was similar in form. In one experiment the heel-pinch reflex was tested every 10 sec and it was extinguished after 40 sec inhalation. Ether and chloroform vapour were used in two experiments only, they gave results similar to trichlorethylene. In four trials two or more type 1 anaesthetics were given in sequence to make sure that the same fibres partook in the responses to the different anaesthetics. The acceleration of the tonic

discharge in a gamma fibre during inhalation of ethyl chloride is shown in Fig. 5 and plotted graphically in Fig. 6.

*Inhalation anaesthetics, type 2: bromochlorotrifluoroethane.* This anaesthetic was found to be very rapid in its action on the gamma reflexes but it did not produce a phase of tonic excitation. When it was given in a similar way

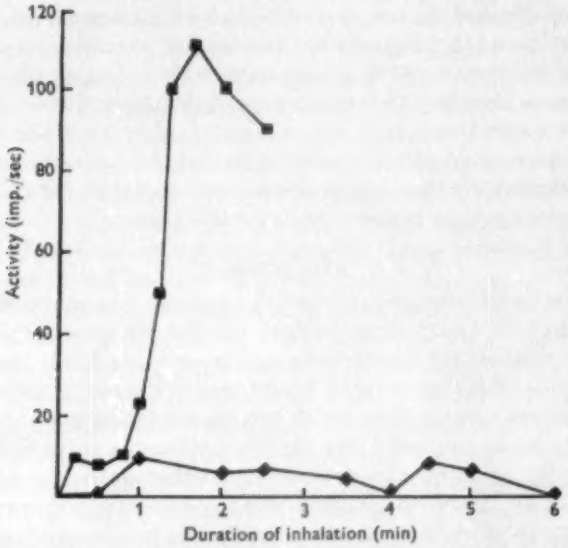



Fig. 6. Activity in a gamma fibre in an intramuscular nerve branch in the medial gastrocnemius muscle. ■, the response to ethyl chloride vapour; ◆, the activity in the same fibre during the inhalation of a mixture of 90 % nitrous oxide and 10 % oxygen.

to trichlorethylene (that is, it was allowed to evaporate from cotton-wool in the open-ended box containing the head of the animal), it extinguished the gamma reflexes within half a minute, and there was no phase of excitation. On return to air breathing the heel-pinch reflex reappeared after 4 min and the ear-pinch and head-shake reflexes after 6 min. When the animal was breathing a 4% (v/v) air mixture and the heel-pinch reflex was tested every 10 sec, it was found that the reflex was affected after 20 sec and extinguished after 80 sec. There was no phase of excitation.

*Nitrous oxide.* This anaesthetic was tried in three concentrations on three animals, namely 100%, 90% nitrous oxide + 10% oxygen, 80% nitrous oxide + 20% oxygen. When it was given as the pure gas, while recording from a fibre that had shown a response to ethyl chloride there was no tonic response and respiratory arrest occurred after 2 min inhala-

tion. At this point there was head-shake movement but no gamma excitation and a reduced heel-pinch response. After recovery the gamma response to anoxia produced with nitrogen was tested and excitation occurred after 1¼ min. Inhalation of 10% $O_2$ + 90% $N_2O$ produced no tonic excitation but there was an interruption of supraspinal effects; after 5 min inhalation the head-shake reflex had gone and there was no response to ethyl chloride, but the toe- and heel-pinch reflexes remained. Figure 6 shows the response of the same fibre first to ethyl chloride and then, after a pause for recovery, to 90% nitrous oxide + 10% oxygen. The effects of 80% nitrous oxide with 20% oxygen were qualitatively similar. It seemed from these experiments that nitrous oxide interrupted the pathways whereby anoxia, ethyl chloride, and the head-shake and ear-pinch reflexes normally excited the spinal gamma motoneurones, but did not interfere with the spinal gamma response to foot stimulation.

## DISCUSSION

Some species differences in the reflex responses to ear stimulation are evident. In the lightly anaesthetized rat the pinna and the external auditory meatus are the most reflexogenic zones on the head. On the other hand, Diete-Spiff & Pascoe (1959) report that in the rabbit the skin of the ears was the only region from which gamma excitation was not obtained. As has been shown by Granit *et al.* (1952), twisting the pinna in the cat has a potent effect on lumbar gamma activity, either causing acceleration of discharge or an inhibition followed by an acceleration. However, in the present rat experiments ear-twist was ineffective but ear-pinch was always excitatory to the limb gamma efferents, and in some preparations, to alpha fibres as well. In the anaesthetized rat the scratch reflex does not occur after ear-pinch, but slight movements of the ipsilateral forelimb were seen rarely. Sherrington (1917) included the head-shake reflex in his description of the ear reflexes of the cat, but later workers do not appear to have studied its gamma fibre component to the limbs. Certainly one would expect that the oscillatory movements of the head would be accompanied by phasic adjustments of the innervation of limb muscles in order to preserve equilibrium.

The surfaces of the foot which either excite or inhibit the gamma efferents serving the gastrocnemius muscle of the rat are similar to those described by Eldred & Hagbarth (1954) for the cat, except that the excitatory zone in the rat extends rather more on to the sole of the foot. The fact that the sural nerve contains afferents from these two zones of opposite action on the gamma motoneurones of the gastrocnemius muscle suggests that rather variable results will follow the electrical stimulation of the whole nerve trunk. Urethane was used as the basal anaesthetic in

most of the experiments described in this paper, and for this reason care was taken to check whether it was a stimulant to the gamma system, as has been reported in the rabbit by Diete-Spiff & Pascoe (1959). No excitation was seen at any stage, but of course these tests refer only to doses additional to that required to produce light anaesthesia. It is difficult to predict what the level of gamma activity is in the intact unanaesthetized animal. The activity in the decerebrate unanaesthetized preparation is variable, and if rigidity is present the gamma activity may follow the spontaneous changes in rigidity (Hunt, 1951). A feature which was frequently observed in the present study was that as the anaesthesia lightened the level of gamma activity became more labile, was easily influenced by an increasing range of sensory stimulation and in some instances was subject to apparently spontaneous rhythmic shifts of activity level. Thus, it may be that in the intact unanaesthetized animal, the level of gamma activity is very labile, being increased in states of alertness or in anticipation of a movement. The familiar difficulty of distinguishing between small and large motor fibres was present throughout this work. The small fibres could rarely be detected except in very slender nerve filaments. Furthermore, when recording from intramuscular branches the activation of alpha fibres in nearby innervated muscle led to electromyographic activity which could be heard on the loudspeaker and to slight movements which could be seen through the dissecting microscope. This information was useful but not conclusive, since alpha activity does not necessarily begin simultaneously in all parts of the muscle. The use of action-potential size (Hunt, 1951) is satisfactory if one is permitted to suspend judgement in preparations where the ratio of action-potential sizes is small. These situations arise frequently, and there is a need for simple distinguishing tests. It is possible that the use of a quickly-acting volatile anaesthetic such as ethyl chloride may be of value, but first it must be decided whether all small motor fibres are excited, or whether a group of some significance functionally alone is stimulated. Certainly several filaments which had been predicted from other features to be gamma efferents failed to respond to ethyl chloride and trichlorethylene. Another feature which is of interest is the great range of observed fibre response. In some fibres the frequency was doubled before it ceased, in others the effect was much greater, e.g. an increase from zero to 100 imp./sec. If this range in the extent of control is genuine and not due to some uncontrolled variable in the experimental procedure, one might expect that some gammas would be beyond the control entirely.

In Fig. 5 the signal-to-noise ratio is unusually large for a gamma fibre; this is due to the use of a particularly slender nerve filament. This is of importance here to show that it is the record from a single active fibre,

since constancy of action-potential size together with regularity of discharge, if found together for several seconds, exclude the possibility that the record is made up of the activity of two or more fibres. Constancy of action-potential size can only be obtained if there is a large signal-to-noise ratio.

It is not clear from these experiments whether the excitatory effect of the type 1 volatile anaesthetics is due to a direct central action or whether it is due to reflex arousal caused by sensitization of sensory endings. Robertson, Swan & Whitteridge (1956) have shown that the threshold of carotid baroceptors is lowered by inhaled ether, chloroform and trichlorethylene. Whitteridge & Bülbring (1944) found that the sensitivity of pulmonary stretch endings was increased and Matthews (1933) detected an increase in excitability of muscle endings during induction of chloroform anaesthesia. Considerations which do not favour this second possibility are, first, that Schulte, Henatsch & Busch (1959) have shown that an increased inflow from the carotid sinus in the cat causes an inhibition of the lumbar gamma motoneurones, and secondly, that with trichlorethylene the main excitation occurs at a stage when other sensory influences have little effect, i.e. when the depth of anaesthesia interrupted sensory connexions with the gamma system.

SUMMARY

1. A study has been made of motor discharges in alpha and gamma fibres to the hind limb of the lightly anaesthetized rat. A group of reflexes which influence gamma activity is described.

2. Three anaesthetics have been used to produce the light basal anaesthesia, namely urethane, pentobarbitone sodium and thiopentone sodium.

3. The effects of additional doses of anaesthetic, given either by inhalation or injection, on the resting discharge and the reflex excitability of the gamma and alpha fibres has been examined.

4. The inhalation anaesthetics have been divided into two groups. The first includes ethyl chloride, trichlorethylene, ether and chloroform. The second includes bromochlorotrifluoroethane and nitrous oxide. Anaesthetics of the first group cause a tonic excitation of gamma efferents and a simultaneous interruption first of supraspinal and then of spinal reflexes. Anaesthetics of the second group have a similar effect on the reflexes but do not produce a tonic excitation.

I wish to thank Professor G. H. Bell for his interest in this work.

## REFERENCES

ANDREW, B. L. (1959). Reflex activity in small motor fibres from the lumbar spinal cord. *J. Physiol.* **148**, 28–29*P*.

ANDREW, B. L. (1960). The effect of certain anaesthetics on the activity of gamma motoneurones serving the hind limb of the rat. *J. Physiol.* **154**, 24*P*.

DIETE-SPIFF, K. & PASCOE, J. E. (1959). The spindle motor nerves to the gastrocnemius muscle of the rabbit. *J. Physiol.* **149**, 120–134.

ELDRED, E. & HAGBARTH, K.-E. (1954). Facilitation and inhibition of gamma efferents by stimulation of certain skin areas. *J. Neurophysiol.* **17**, 59–65.

GRANIT, R., JOB, C. & KAADA, B. R. (1952). Activation of muscle spindles in pinna reflex. *Acta physiol. scand.* **27**, 161–168.

HUNT, C. C. (1951). The reflex activity of mammalian small-nerve fibres. *J. Physiol.* **115**, 456–469.

HUNT, C. C. & PAINTAL, A. S. (1958). Spinal reflex regulation of fusimotor neurones. *J. Physiol.* **143**, 195–212.

MATTHEWS, B. H. C. (1933). Nerve endings in mammalian muscle. *J. Physiol.* **78**, 1–53.

ROBERTSON, J. D., SWAN, A. A. B. & WHITTERIDGE, D. (1956). Effect of anaesthetics on systemic baroceptors. *J. Physiol.* **131**, 463–472.

SCHULTE, F. J., BUSCH, G. & HENATSCH, H. D. (1959). Antriebssteigerungen lumbaler Extensor-Motorneurone bei Aktivierung der Chemoreceptoren in Glomus caroticum. *Pflüg. Arch. ges. Physiol.* **269**, 580–592.

SCHULTE, F. J., HENATSCH, H. D. & BUSCH, G. (1959). Über den Einfluss der Carotissinus-Sensibilität auf die spinalmotorischen Systeme. *Pflüg. Arch. ges. Physiol.* **269**, 248–263.

SHERRINGTON, C. S. (1917). Reflexes elicitable in the cat from pinna, vibrissae and jaws. *J. Physiol.* **51**, 404–431.

WHITTERIDGE, D. & BÜLBRING, E. (1944). Changes in activity of pulmonary receptors in anaesthesia and their influence on respiratory behavior. *J. Pharmacol.* **81**, 340–359.

*J. Physiol.* (1961), **155**, *pp.* 72–85
*With 4 text-figures*
*Printed in Great Britain*

# BRAIN TEMPERATURE AS A FACTOR IN THE CONTROL OF THERMAL POLYPNOEA IN THE OX (*BOS TAURUS*)

BY J. D. FINDLAY AND D. L. INGRAM
*From the Hannah Dairy Research Institute, Kirkhill, Ayr*

(*Received* 29 *July* 1960)

When cattle are exposed to high environmental temperatures the heat content of the body is regulated partly by an increase in respiratory rate (Brody, 1948). Some evidence (Bligh, 1957*a*) suggests that in calves the stimulus for this increase in the respiratory rate arises from stimulation of the periphery rather than from a rise in the temperature of the blood. In Bligh's experiments it was demonstrated that when calves are suddenly exposed to a high temperature and humidity the increase in the frequency of breathing begins before there is any rise in the temperature of the blood in the carotid artery. Similar experiments by Bazett (1927), who exposed dogs to solar radiation, had led to the same conclusion, that panting is initiated by peripheral stimulation. Nevertheless, it is known (Ranson, 1940) that the brain contains regulatory centres essential for the maintenance of homoeothermy. Thus, electrical stimulation or local heating of the anterior region of the hypothalamus is accompanied by an increase in the rate of respiration in the cat (Magoun, Harrison, Brobeck & Ranson, 1938) and in the goat (Andersson, Grant & Larsson, 1956; Andersson & Persson, 1957).

The respiratory rate, in addition to being influenced by changes in the concentration of carbon dioxide in the blood, can thus be stimulated by increase in temperature either at the periphery or centrally. But the relative importance of these stimuli in the initiation and maintenance of panting is not clear. Nor is it certain that all mammals behave in a similar manner. Hemingway, Rasmussen, Wikoff & Rasmussen (1940) and Ström (1950) failed to cause a change in respiratory rate by heating the hypothalamus in the dog. Using another experimental technique, Forster & Ferguson (1952) measured the hypothalamic temperature of seven unanaesthetized cats during exposure to high temperatures. They found that in five animals the rise in respiratory rate was correlated with a rise in hypothalamic temperature, while in the remaining two the rate of breathing increased without a change in the temperature of the brain.

No information is available about changes in the temperature of the brain of the ox during heat stress, nor has the heat sensitivity of the brain

been investigated in this species. Among several possibilities which need to be considered are: (i) that the increase in respiratory rate on exposure to heat stress may be initiated and controlled purely by stimuli which originate peripherally; (ii) that panting may be initiated by stimulation acting at the periphery, but that the precise magnitude of the increase in the rate of breathing may be determined by changes in the temperature of the brain; (iii) that, while the temperature of the body may be regulated entirely by peripheral stimulation under moderate heat stress, more severe conditions may involve the heat sensitivity of the brain.

In the present study the temperatures of the blood in the carotid artery and of various parts of the brain have been measured under several environmental conditions. In addition, information has been obtained about the change in respiratory rate which occurs when the brain is heated locally. It was also possible to make observations on the effect of the rise in blood temperature which occurs after feeding.

## METHODS

Five Ayrshire bull calves aged between 6 and 10 months were used. They were exposed in a climatic room (Findlay, McLean & Bennet, 1959) to three environmental conditions, (i) 7·5° C dry bulb (DB) temperature 4·5° C wet bulb (WB) temperature, (ii) 18° C DB, 15° C WB, (iii) 35° C DB, 28° C WB. The animal was placed in a stall equipped with eight 250 W infra-red lamps placed four on each side and approximately 30 cm from the animal. The calves were first exposed to the room conditions, stated above, for 1 hr. The infra-red lights were turned on and observations were made for a further 1½–3 hr. The lights were then turned off and the experiment continued for a further hour. Observations of temperature and respiratory rate were made every ten minutes.

In another experiment the animals were starved for 24 hr before exposure in the climatic room. After the observations detailed below had been made for 1 hr the animals were provided with food and allowed to eat for about an hour. The food was then removed and measurements of temperature and respiratory rate were again taken for 1 hr.

The brain was heated locally during exposure to each of the environmental conditions. The rectal temperature was determined by means of a 40 s.w.g. copper-constantan thermocouple inserted 10 cm into the rectum. The temperature of the blood at the bicarotid junction was measured by means of an implanted 25 s.w.g. copper-constantan thermocouple, according to the method previously used in this Institute by Bligh (1957*a*, *b*).

Temperatures in the brain were observed by means of an apparatus consisting of two thermocouples 3 cm apart. A wooden rod 2·5 mm in diameter and approximately 8 cm long was used to support the wires. The end of the apparatus carried a coil of wire, having a resistance of approximately 7 Ω and designed to carry a current of 0·4 A, which was used as a heater. One copper-constantan thermocouple was attached to a copper sheath round the coil of wire and the other to the side of the rod. The whole apparatus was covered with 'Durofix' cement. With the aid of a simple stereotaxic instrument the apparatus was inserted into the brain through a small drill hole in the top of the skull. The thermocouple and heater wires were led to a Perspex plate on which contacts were mounted. The plate was attached to the skull with screws. The operations were carried out aseptically under 'Fluothane' (1.1.1 trifluoro-2 chloro-2 bromo ethane-I.C.I.) anaesthesia.

The rate of breathing was determined by means of the instrument described by Nisbet

(1958). In certain experiments the volume of air taken at each breath was measured by means of a pneumotachometer (Nisbet, 1956). Tidal volumes were measured for 1 min.

The temperature of the skin on the lumbar region was determined with 40 s.w.g. copper-constantan thermocouples attached to the shaved skin with thin rubber patches and rubber cement (Beakley & Findlay, 1955*a*).

*Controls.* All animals were observed in each environmental condition during the week before the experiment to determine whether the implantation of thermocouples caused any gross changes in the control of body temperatures. The animals were observed in the climatic room two or three days after the operation.

*Post-mortem examination.* The position of the thermocouple in the carotid artery was checked at post-mortem examination. The brain was removed and fixed in 10% formol saline. The position of the thermocouples was then determined by section of the brain. In those animals in which the heater was placed within the heat-sensitive part of the hypothalamus, the region near the heater was examined histologically to determine the extent of the damage to the surrounding tissues.

## RESULTS

### *Effect of surgery on the control of body temperature*

Comparison of the animals' reactions to heat before and after operation revealed that only calf D was affected by the implantation of thermocouples. The body temperature of this animal was above the control value during the first few experiments after the operation, but later returned to normal. Calf E suffered damage to the radial nerve, due to slipping on a wet floor, and was unable to stand properly. Experiments with this animal had therefore to be restricted. No other abnormalities were noted in any of the animals. At post-mortem examination the tip of the thermocouple in the carotid artery was found to be at the bicarotid junction or in the innominate artery in all the animals. The positions of the brain thermocouples are shown in Fig. 1.

### *Exposure to an environment of 7·5° C DB and 4·5° C WB*

Records are available for ten experiments on four animals. The general behaviour of the respiratory rate and of the temperatures of the blood, brain and skin when the calf was irradiated with infra-red heat is illustrated in Fig. 2. This figure shows the mean increase in temperature at 10 min intervals computed from the results of ten experiments. Changes in temperature and respiratory rate were calculated by reference to the mean value obtained over the first 55 min in each experiment. Immediately after turning on the heaters the respiratory rate rose, and continued to rise for some 50 min, and then became stable at a value some 20–25 respirations/min above the mean normal value obtained in the period previous to heating. The rise in respiratory rate was accompanied by an immediate rise in skin temperature. No rise in the temperature of the brain occurred until some 15 min after the heaters had been turned on. The temperature of the brain then rose and became stable at about 0·5° C

above the control value. There was no immediate rise in the temperature of the blood in the carotid artery; in fact, there was a slight fall after the first 5 min of exposure to heat. The blood temperature rose and attained a constant level some 35 min after the heaters were turned on. The increase

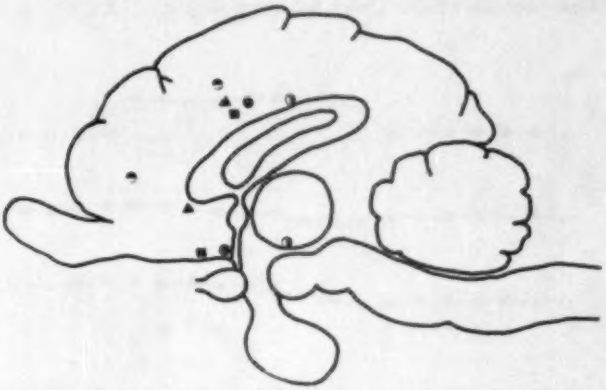


Fig. 1. Section of brain showing position of thermocouples. ◓ = calf A, ◑ = calf B, ◒ = calf C, ▲ = calf D, ■ = calf E. The most ventral point in the diagram represents the position of thermocouple I and the heater, and the dorsal point gives the position of thermocouple II.

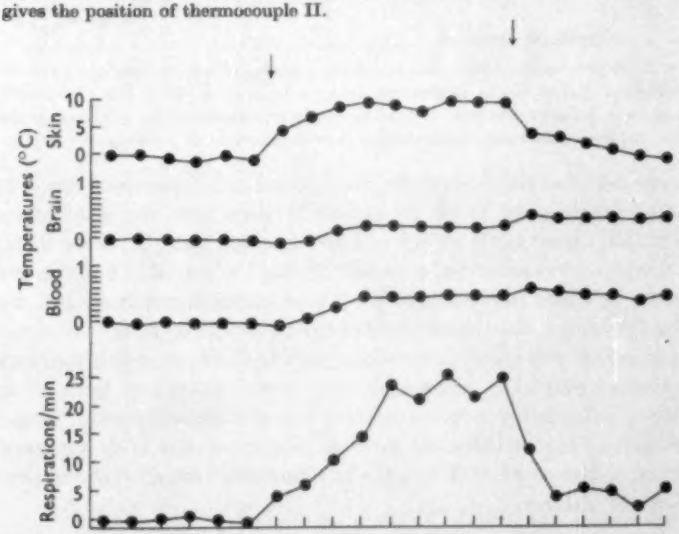



Fig. 2. Mean changes in temperature and respiratory rate on exposure to infra-red irradiation (between the arrows) in an environment of 7·5° C DB, 4·5° C WB. Changes in temperature and respiratory rate were calculated by reference to the mean value obtained over the first hour in each experiment. Time marker, 10 min.

in temperature was greater in the blood than in the brain. On turning off the heaters there was an immediate fall in respiratory rate and in skin temperature, but the temperatures of both the brain and the blood in the carotid artery rose. The temperature of the brain then remained constant, but the temperature of the blood fell very slightly.

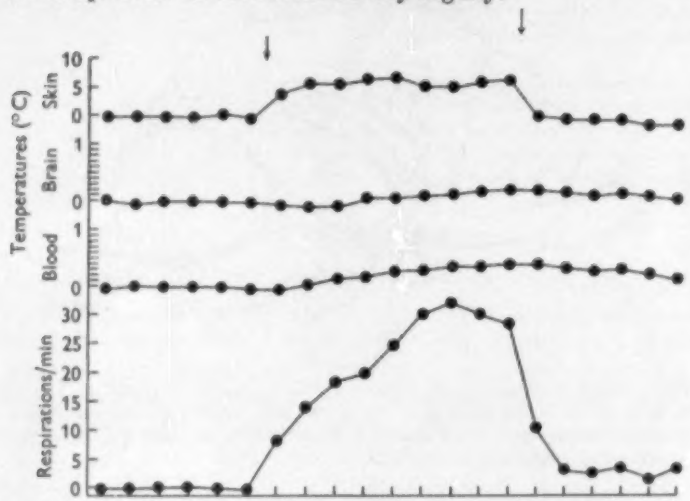



Fig. 3. Mean changes in temperature and respiratory rate on exposure to infra-red irradiation (between the arrows) in an environment of 18° C DB, 15° C WB. Changes in temperature and respiratory rate were calculated by reference to the mean value obtained over the first hour in each experiment. Time marker, 10 min.

A more detailed account of the changes in the temperature of the blood and brain is given in Table 1. It will be seen that the fluctuations in temperature varied both within and between animals. In some instances these temperatures remained constant during the period of heating or even declined. In other experiments the blood temperature increased during heating. However, the rise of blood temperature after the infra-red heaters were turned off was always observed. During the first two exposures after the operation animal D had a high body temperature and by comparison with other animals the respiratory rate was also above normal. Exposure of this animal to the infra-red heaters resulted in the body temperature increasing to above 40° C. When the heaters were turned off the respiration rate did not decline.

*Exposure to an environment of 18° C DB and 15° C WB*

The same animals were used in nine experiments at 18° C. The general pattern of temperature and respiratory rate during infra-red irradiation

TABLE 1. Changes in temperature (° C) of blood and brain and in respiratory rate during infra-red irradiation at environmental temperatures of 7·5° C DB and 4·5° C WB (means ± S.E.). Readings taken every 10 min

| Animal | Expt. | Temperatures of blood in carotid artery — Irradiation: Before | During | After | Temperature of brain at thermocouple I* — Irradiation: Before | During | After |
|---|---|---|---|---|---|---|---|
| A | 1 | 39·1 ± 0·003 | 39·0 ± 0·001 | 39·1 ± 0·006 | 39·2 ± 0·002 | 39·1 ± 0·003 | 39·3 ± 0·007 |
| | 2 | 38·5 ± 0·002 | 38·7 ± 0·002 | 38·8 ± 0·007 | 38·7 ± 0·003 | 38·9 ± 0·002 | 39·1 ± 0·007 |
| | 3 | 38·3 ± 0·002 | 38·5 ± 0·007 | 38·8 ± 0·003 | 38·4 ± 0·002 | 38·6 ± 0·007 | 38·9 ± 0·004 |
| B | 1 | 39·2 ± 0·002 | 39·1 ± 0·002 | 39·2 ± 0·001 | 39·2 ± 0·001 | 39·0 ± 0·003 | 39·3 ± 0·002 |
| | 2 | 37·7 ± 0·003 | 38·6 ± 0·266 | 38·8 ± 0·002 | 37·9 ± 0·003 | 38·7 ± 0·235 | 39·0 ± 0·002 |
| C | 1 | 37·9 ± 0·005 | 38·6 ± 0·008 | 38·7 ± 0·100 | 38·1 ± 0·004 | 38·6 ± 0·009 | 38·8 ± 0·100 |
| | 2 | 37·6 ± 0·005 | 37·9 ± 0·200 | 38·1 ± 0·007 | 37·7 ± 0·000 | 37·7 ± 0·100 | 38·4 ± 0·000 |
| D | 1 | 39·9 ± 0·002 | 40·6 ± 0·500 | 40·7 ± 0·100 | 40·0 ± 0·002 | 40·3 ± 0·100 | 40·5 ± 0·007 |
| | 2 | 40·0 ± 0·000 | 40·9 ± 0·200 | 41·1 ± 0·007 | 40·1 ± 0·003 | 40·6 ± 0·009 | 41·0 ± 0·004 |
| | 3 | 38·6 ± 0·002 | 39·0 ± 0·009 | 39·2 ± 0·004 | 38·8 ± 0·002 | 38·8 ± 0·004 | 39·2 ± 0·002 |

| Animal | Expt. | Temperature of brain at thermocouple II* — Irradiation: Before | During | After | Respiration (min) — Irradiation: Before | During | After |
|---|---|---|---|---|---|---|---|
| A | 1 | 39·2 ± 0·002 | 39·2 ± 0·002 | 39·3 ± 0·006 | 18·4 ± 1·2 | 43·8 ± 3·3 | 19·8 ± 0·7 |
| | 2 | 38·7 ± 0·002 | 38·9 ± 0·002 | 39·1 ± 0·007 | 12·2 ± 0·4 | 40·4 ± 3·3 | 18·0 ± 3·0 |
| | 3 | 38·4 ± 0·002 | 38·7 ± 0·007 | 39·0 ± 0·002 | 11·7 ± 0·5 | 34·9 ± 2·4 | 13·8 ± 0·3 |
| B | 1 | 39·1 ± 0·002 | 39·0 ± 0·007 | 39·2 ± 0·001 | 13·9 ± 0·4 | 32·1 ± 2·3 | 17·4 ± 1·2 |
| | 2 | — | — | — | 13·9 ± 1·1 | 45·1 ± 2·0 | 17·2 ± 2·4 |
| C | 1 | 38·0 ± 0·004 | 38·7 ± 0·100 | — | 16·4 ± 0·5 | 33·7 ± 4·0 | 21·1 ± 1·3 |
| | 2 | 37·8 ± 0·003 | 38·5 ± 0·200 | 38·6 ± 0·000 | 29·6 ± 2·5 | 34·7 ± 2·5 | 32·0 ± 2·0 |
| D | 1 | 40·0 ± 0·002 | 40·4 ± 0·008 | 40·5 ± 0·009 | 45·3 ± 1·3 | 57·2 ± 1·9 | 58·0 ± 3·3 |
| | 2 | 40·2 ± 0·002 | 40·6 ± 0·100 | 41·0 ± 0·004 | 31·4 ± 1·0 | 43·6 ± 3·5 | 42·8 ± 2·3 |
| | 3 | 38·8 ± 0·003 | 38·7 ± 0·003 | 39·2 ± 0·003 | 20·6 ± 0·9 | 36·3 ± 2·4 | 26·4 ± 1·0 |

* See Fig. 1 for position of thermocouples.

is shown in Fig. 3. The responses of the calves were similar to those observed at the lower temperature. The increase in the temperature of the blood was again greater than that of the brain. The rise in skin temperature was not as great as at 7·5° C, but the increase in respiratory rate was greater than that at 7·5° C (Tables 1 and 2). At the end of the period of heating the body temperatures did not rise as in the experiments at 7·5° C but fell very gradually. The temperature of the skin however fell rapidly to the control value. The respiratory rate also fell rapidly but again remained slightly higher than the control value.

The results of individual experiments are shown in Table 2. As in the experiments at 7·5° C, in some instances the body temperatures remained steady during irradiation, but in others the temperature actually fell (B (ii)). The temperature of animal D, again, increased during the period of heating, in two instances to over 40° C. In these experiments the fall in respiratory rate after the heaters were turned off was slight. It will be seen from Tables 1 and 2 that in all animals the difference in temperature between the two thermocouples in the brain was very small.

*Exposure to an environment of 35° C DB and 28° C WB*

In these experiments the animals were exposed to infra-red irradiation until the rectal temperature had reached 41·8–42·0° C. This temperature was regarded as the highest to which the calf could safely be subjected.

Both blood temperature and respiratory rate tended to rise slowly during the period previous to infra-red heating. On exposure to infra-red irradiation the increases in respiratory rate and also in the temperatures of the brain and the blood in the carotid artery were immediate. In these experiments there was no tendency for the blood temperatures to fall on exposure to additional heat.

When the animals were allowed to cool in the room at 35° C without infra-red irradiation the temperature of the body declined immediately but slowly. In five out of eight experiments, however, there was a marked rise in respiratory rate as the body cooled, after which respiration rate declined. In the remaining three instances the rise was less obvious.

*Effect of increased body temperature after feeding*

Observations of temperature and respiratory rate were made during the whole experiment, but it was found that the movements made by the animal while it fed were transmitted to the respiratory counter. Comparisons of respiratory rate had therefore to be restricted to the hour before feeding and the hour after food had been removed. The results obtained are summarized in Table 3. The temperature of the brain and of the blood in the carotid artery increased towards the end of the period

TABLE 2. Changes in temperature (° C) of blood and brain and in respiratory rate during infra-red irradiation at environmental temperatures of 18° C DB and 15° C WB (Means ± S.E.). Readings taken every 10 min

| Animal | Expt. | Temperature of blood in carotid artery — Irradiation: Before | During | After | Temperature of brain at thermocouple I* — Irradiation: Before | During | After |
|---|---|---|---|---|---|---|---|
| A | 1 | 38·9 ± 0·002 | 39·0 ± 0·006 | 39·2 ± 0·009 | 39·2 ± 0·002 | 39·2 ± 0·006 | 39·4 ± 0·007 |
| | 2 | 38·6 ± 0·002 | 38·3 ± 0·002 | 38·8 ± 0·002 | 38·7 ± 0·002 | 38·5 ± 0·003 | 38·9 ± 0·003 |
| B | 1 | 39·2 ± 0·000 | 39·2 ± 0·002 | 39·2 ± 0·005 | 39·3 ± 0·003 | 39·3 ± 0·002 | 39·2 ± 0·004 |
| | 2 | 38·4 ± 0·001 | 38·7 ± 0·002 | 39·0 ± 0·002 | 38·6 ± 0·002 | 38·9 ± 0·002 | 39·1 ± 0·003 |
| C | 1 | 38·9 ± 0·006 | 39·1 ± 0·006 | 38·9 ± 0·009 | 38·9 ± 0·003 | 39·0 ± 0·002 | 39·0 ± 0·005 |
| | 2 | 39·0 ± 0·001 | 39·3 ± 0·010 | 38·9 ± 0·006 | 38·9 ± 0·003 | 39·0 ± 0·001 | 39·0 ± 0·004 |
| D | 1 | 39·2 ± 0·000 | 39·5 ± 0·107 | 39·7 ± 0·005 | 39·4 ± 0·004 | 39·4 ± 0·103 | 39·6 ± 0·003 |
| | 2 | 39·8 ± 0·001 | 40·7 ± 0·154 | 40·8 ± 0·008 | 40·0 ± 0·002 | 40·3 ± 0·114 | 40·5 ± 0·007 |
| | 3 | 39·6 ± 0·000 | 40·3 ± 0·128 | 40·3 ± 0·009 | 39·5 ± 0·006 | 39·7 ± 0·112 | 39·9 ± 0·009 |

| Animal | Expt. | Temperature of brain at thermocouple II* — Irradiation: Before | During | After | Respiration (min) — Irradiation: Before | During | After |
|---|---|---|---|---|---|---|---|
| A | 1 | 39·1 ± 0·002 | 39·2 ± 0·005 | 39·3 ± 0·004 | 20·6 ± 0·9 | 89·8 ± 8·2 | 23·7 ± 3·7 |
| | 2 | 38·7 ± 0·002 | 38·6 ± 0·003 | 39·0 ± 0·002 | 16·0 ± 0·3 | 76·2 ± 4·2 | 15·4 ± 0·7 |
| B | 1 | 39·2 ± 0·002 | 39·2 ± 0·002 | 39·1 ± 0·003 | 18·0 ± 0·4 | 72·0 ± 6·8 | 22·6 ± 4·1 |
| | 2 | — | — | — | 15·1 ± 0·9 | 91·0 ± 6·1 | 21·8 ± 2·3 |
| C | 1 | 38·9 ± 0·006 | 39·0 ± 0·002 | 39·0 ± 0·005 | 32·8 ± 1·5 | 63·6 ± 2·8 | 34·8 ± 2·2 |
| | 2 | 39·0 ± 0·003 | 39·0 ± 0·001 | 39·0 ± 0·002 | 30·3 ± 0·7 | 64·1 ± 2·3 | 33·6 ± 2·0 |
| D | 1 | 39·4 ± 0·004 | 39·4 ± 0·114 | 39·6 ± 0·009 | 34·5 ± 1·0 | 40·6 ± 2·7 | 38·3 ± 0·9 |
| | 2 | 40·0 ± 0·000 | 40·4 ± 0·117 | 40·6 ± 0·008 | 54·9 ± 2·3 | 77·7 ± 5·9 | 70·7 ± 4·6 |
| | 3 | 39·5 ± 0·004 | 39·8 ± 0·112 | 40·0 ± 0·008 | 42·9 ± 2·1 | 78·8 ± 5·5 | 63·3 ± 4·8 |

* See Fig. 1 for position of thermocouples.

allowed for feeding and then declined slowly after the food was removed. The values of the respiratory rates recorded after feeding were compared with those obtained during the control period by means of a $t$ test. The analysis revealed that when the rise in the temperature of the brain was more than 0·5° C the change in respiratory rate was statistically significant ($P < 0·001$). An increase of less than 0·5° C was associated with a statistically significant increase in the respiratory rate in only one instance.

TABLE 3. Rise in the temperature of the brain and the respiratory rate after feeding

| | | Brain temperature (° C; thermocouple I) | | | Respiratory rate resp./min | |
|---|---|---|---|---|---|---|
| Animal | Expt. | Before feeding | After feeding | Mean rise | Mean rise | Significance of rise ($P$) |
| A | 1 | 37·86 ± 0·003 | 38·85 ± 0·005 | + 0·99 | + 2·5 | 0·01–0·001 |
| B | 1 | 38·40 ± 0·000 | 39·14 ± 0·004 | + 0·54 | + 4·56 | < 0·001 |
| | 2 | 38·40 ± 0·000 | 38·97 ± 0·003 | + 0·57 | + 3·54 | < 0·001 |
| | 3 | 38·47 ± 0·003 | 38·84 ± 0·003 | + 0·37 | + 1·42 | ≏ 0·2 |
| C | 1 | 38·58 ± 0·010 | 39·54 ± 0·003 | + 0·96 | + 10·10 | < 0·001 |
| D | 1 | 39·23 ± 0·005 | 39·29 ± 0·003 | + 0·06 | − 2·29 | 0·2–0·1 |
| | 2 | 39·28 ± 0·004 | 39·39 ± 0·004 | + 0·16 | − 0·72 | 0·3–0·2 |
| | 3 | 38·53 ± 0·002 | 38·69 ± 0·003 | + 0·11 | + 2·08 | 0·01–0·001 |

*The effect of local heating of the hypothalamus*

In two animals the heater was placed within the heat-sensitive region of the hypothalamus. Heaters placed in other regions of the brain had no obvious effect on the animal.

*Animal E.* As already mentioned, this animal had a damaged radial nerve and only a limited number of experiments were performed. The animal maintained a steady body temperature in an environment of 20° C DB and 19·5° C WB and also at 9·5° C DB and 8·4° C WB. Under both conditions the temperature of the ear was only slightly above that of the environment. Local heating of the brain caused an increase in the respiratory rate to about twice the control value after 2–3 min, and an increase in the temperature of the ear to 35° C. After heating the brain for 10 min, during which time the respiratory rate was maintained at the increased value, the body temperature fell by 0·5° C.

*Animal C.* This calf also responded to local heating of the brain by an increase in the respiratory rate, but no effect was observed on the temperature of the ear. The increase in respiratory rate, and the accompanying fall in body temperature when the brain was heated in an environmental temperature of 7·5° C, are illustrated in Fig. 4. At 18° C heating of the brain was again accompanied by an increase in respiratory rate and a fall in body temperature both with and without infra-red irradiation.

On exposure to 35° C the body temperature increased slowly as in previous experiments. At temperatures of 39·5–39·9° C local heating of the hypothalamus resulted in an increase in the rate of respiration and a decrease in tidal volume (Table 4). After the temperature of the blood had

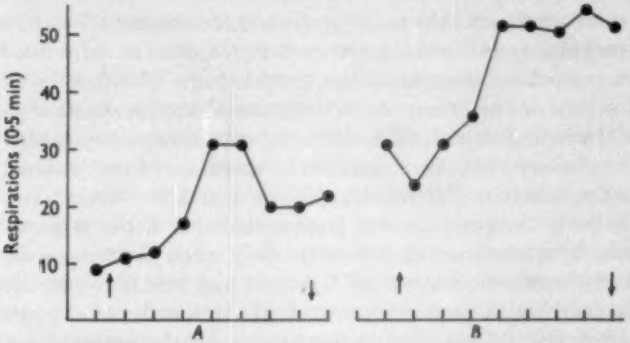



Fig. 4. Effect of local heating of the hypothalamus on respiratory rate. ↑ heater switched on, ↓ end of heating. 7·5° C DB, 4·5° C WB, *A*, without infra-red irradiation; temperature of blood fell from 38·3 to 37·0° C during heating. *B*, with infra-red irradiation; temperature of blood fell from 38·6 to 38·0° C during heating. Time marker, half minutes.

TABLE 4. Changes in respiratory rate and tidal volume during local heating of the hypothalamus at various blood temperatures

(Measurements were taken immediately before, during and after heating and the mean change was computed.)

| Expt. 1 | | | Expt. 2 | | |
|---|---|---|---|---|---|
| Temperature of blood (° C) | Respirations (resp./min) | Tidal volume (l.) | Temperature of blood (° C) | Respirations (resp./min) | Tidal volume (l.) |
| 39·9 | +23·5 | −0·07 | 39·5 | + 5 | −0·02 |
| 40·1 | −13·0 | +0·26 | 39·9 | +24 | −0·17 |
| 40·2 | − 2·5 | +0·14 | 40·6 | + 9 | +0·18 |
| 40·2 | − 6·5 | +0·13 | 40·8 | − 4·5 | +0·07 |
| 40·1 | − 6·0 | +0·16 | 40·8 | + 2·5 | +0·01 |
| — | — | — | 41·3 | + 2 | +0·28 |
| — | — | — | 41·4 | + 1 | +0·13 |
| — | — | — | 41·5 | −10 | +0·21 |

increased to above 40° C, however, the respiration rate decreased slightly in most instances and the tidal volume always increased. This occurred in two separate experiments. But at the end of the second series of observations the animal no longer responded to heating of the brain at any environmental temperature, and it was concluded that damage had been caused in the region of the heater. Histological examination of the hypothalamus revealed that lesions had been produced in both animals. The

position of the heater was marked by a cavity, the sides of which were necrotic. In both animals the heater had been placed in the mid line, and damage to the lateral regions of the hypothalamus had been avoided.

## DISCUSSION

The results indicate that under moderate environmental conditions the rise in respiratory rate, which occurred in response to infra-red heating, was not related to changes in the temperatures of either blood in the carotid artery or the brain. At environmental temperatures of both 7·5° and 18° C irradiation with infra-red light was accompanied by an increase in the respiratory rate. But often the temperature of the brain and blood remained constant or fell slightly (Tables 1 and 2). Even when the increase in body temperature was greater than 0·1° C the respiratory rate fell markedly when irradiation ceased. Only when the mean temperature of the body increased to about 40° C did the high rate of respiration persist after the infra-red heaters were turned off. In a series of experiments on sheep, Bligh (1959) found that in this species also the respiratory rate may in some instances rise steadily for 1 hr without any change in the mean temperature of the body. On the other hand it has been demonstrated in the present study that the rise in the blood temperature which occurred after feeding was accompanied by only a slight increase in respiratory rate.

Experiments by Forster & Ferguson (1952) lend some support to the view that the temperature of structures which lie deep in the body are of importance in the control of heat-loss mechanisms. They introduced ice-cold water into the stomachs of cats subjected to heat stress and observed that the respiratory rate decreased immediately. It is, however, quite possible that the stomach wall contains receptors which were stimulated by the sudden change in temperature. In any event it may be pertinent that these observations relate to animals whose body temperature was considerably above the normal range.

From the work of Bligh (1957*a*, 1959), and the present study, it appears most probable that when the body temperature is within the normal range the stimulus for an increased respiratory rate acts almost entirely at the periphery. The magnitude of the increase in respiratory rate must thus be governed by some factor other than the mean temperature of either the body or the brain. One possibility is that the rate of heat loss from the body surface is of importance in this respect.

The experiments conducted in environments of 7·5 and 18° C yield two further points of interest. One is that at the end of the period of infra-red irradiation, when the skin temperature had returned to the control value, the rate of respiration did not decline completely to the control value, but continued at a rate of about 5 respirations/min above it. If it is assumed

that this slight increase has any physiological significance it may be related to the small persisting rise in the temperature of the brain. It is of interest to note in this connexion that the increase in respiratory rate after feeding was of the same order as that observed at the end of the period of heating, and that the rises in the mean temperatures of the body were also similar. The change in the respiratory rate after feeding may be related to a change in the demand for oxygen, respiratory stimulation from joints, or specific dynamic action of the food, but the possibility that it is directly related to the rise in body temperature cannot be ignored.

The second point which arises from these experiments is that in those instances where the temperature of the body increased to about 40° C the high rate of respiration persisted even after heating was stopped.

Local heating of the hypothalamus of the calf also caused a rise in the respiratory rate but it is very probable that the temperatures needed to produce these effects were above 40° C. In those studies in which the rise in temperature was measured (Magoun *et al.* 1938; Lim & Goodins, 1955) no marked increase in respiratory rate was observed until the temperature reached 42–43° C. Such temperatures may, however, be reached under extreme heat stress in the calf.

It is therefore suggested that the increase in the rate of respiration in response to heating has two components. One depends on peripheral stimulation and is of major importance when the mean temperature of the body is within the normal range. The other, which is related to the temperature of the brain, is of little account under moderate conditions of heating, but assumes much greater significance when the temperature of the body rises above about 40° C. The second component mentioned above is complicated by at least two factors. The first is that, as recently demonstrated by Holmes, Newman & Wolstencroft (1958), not only does local heating of the hypothalamus cause a rise in the respiratory rate, but so also does heating of structures in the hind brain. A rise in the temperature of the brain thus influences respiration rate by its effect on more than one centre.

Findlay (1957) showed that when the rectal temperature reaches 40·5° C there is a sudden decrease in the respiratory rate and a rise in both the tidal and minute volumes. In his opinion the change in respiratory rate suggests that the danger from hypothermia takes precedence over the danger from alkalosis which develops in these animals. As demonstrated by Beakley & Findlay (1955*b*), Findlay (1957) and Bianca (1958), and confirmed in the present study, the respiratory rate increases when the body begins to cool from 42° C. In the experiments of these previous workers, however, only the rectal temperatures were recorded, and the fall in the temperature of the body occurred after the respiratory rate began

to rise. The results of Bligh (1957*b*) which have been confirmed in the present study, indicate, however, that the temperature recorded in the rectum lags behind that of the blood during cooling and that the temperature of the blood falls immediately. The likelihood is that, in Findlay's and Bianca's experiments also, the fall in the blood temperature was immediate. It is possible that under these conditions, when the temperature of the body is falling, the danger from alkalosis takes precedence over hyperthermia.

If the changes in the rate of respiration and in the tidal volume are controlled, at least in part, by the hypothalamus, then it is likely that the reaction of the centre to heat depends on whether the body as a whole is gaining or losing heat. Some evidence which suggests that the reaction of the hypothalamus to heat does depend on the temperature of the rest of the body is provided by the present experiments in which the brain was heated while the blood temperature was above and also below 40° C.

## SUMMARY

1. The temperature of the brain and of the blood in the carotid artery has been measured in calves.

2. In environments at 7·5° C DB and 18° C DB heating with infrared irradiation was accompanied by a rise in the respiratory rate but body temperature at first fell slightly before beginning to rise. In an environment at 35° C there was no fall in body temperature on exposure to infrared irradiation, but respiratory rate increased.

3. When irradiation ceased, respiratory rate declined to almost the control value provided the body temperature was below 40° C. At body temperatures above 40° C removal of the irradiation had no immediate effect on the respiratory rate.

4. A rise in body temperature of 0·5° C after feeding resulted in only a very slight increase in the respiratory rate.

5. Local heating of the hypothalamus caused an increase in respiratory rate and a decrease in tidal volume when the body temperature was below 40° C. There is some evidence, however, that at higher body temperatures heating of the hypothalamus caused a decrease in respiratory rate and an increase in tidal volume.

6. It is suggested that when the body temperature is within the normal range thermal polypnoea is controlled by stimulation at the periphery. At body temperatures above about 40° C stimulation of central receptors appears to become of major importance.

The authors are grateful to Dr F. R. Bell, Royal Veterinary College, for his helpful criticisms during the preparation of this paper; to Miss D. McLelland and Mr R. Mabon for their skilled technical assistance and to Mr W. Nisbet who manufactured the thermo-

junctions. This work was carried out in a climatic laboratory built with the aid of a grant by the Colonial Office from the Colonial Development and Welfare Fund.

## REFERENCES

ANDERSSON, B. & PERSSON, N. (1957). The pronounced hyperthermia elicited by prolonged stimulation of the 'Heat Loss Centre' in unanaesthetized goats. *Acta physiol. scand.* **41**, 277–282.

ANDERSSON, B., GRANT, R. & LARSSON, S. (1956). Central control of heat loss mechanisms in the goat. *Acta physiol. scand.* **37**, 261–280.

BAZETT, H. C. (1927). Physiological responses to heat. *Physiol. Rev.* **7**, 531–599.

BEAKLEY, W. R. & FINDLAY, J. D. (1955*a*). The effect of environmental temperature and humidity on the skin temperature of Ayrshire calves. *J. agric. Sci.* **45**, 353–364.

BEAKLEY, W. R. & FINDLAY, J. D. (1955*b*). The effect of environmental temperature and humidity on the respiration rate of Ayrshire calves. *J. agric. Sci.* **45**, 452–460.

BIANCA, W. (1958). The relation between respiratory rate and heart rate in the calf subjected to severe heat stress. *J. agric. Sci.* **51**, 321–324.

BLIGH, J. (1957*a*). The initiation of thermal polypnoea in the calf. *J. Physiol.* **136**, 413–419.

BLIGH, J. (1957*b*). The relationship between the temperature in the rectum and of the blood in the bicarotid trunk of the calf during exposure to heat stress. *J. Physiol.* **136**, 393–403.

BLIGH, J. (1959). The receptors concerned in the thermal stimulus to panting in sheep. *J. Physiol.* **146**, 142–151.

BRODY, S. (1948). Environmental physiology with special reference to domestic animals. 1. Physiological backgrounds. *Res. Bull. Mo. agric. Exp. Sta.* **423**.

FINDLAY, J. D., MCLEAN, J. A. & BENNETT, R. D. (1959). A climatic laboratory for farm animals. *Heat. Vent. Engr.* **33**, 95–100.

FINDLAY, J. D. (1957). The respiratory activity of calves subjected to thermal stress. *J. Physiol.* **136**, 300–309.

FORSTER, R. E. & FERGUSON, T. B. (1952). Relationship between hypothalamic temperature and thermoregulatory effectors in unanaesthetized cat. *Amer. J. Physiol.* **169**, 255–269.

HEMINGWAY, A., RASMUSSEN, T., WIKOFF, H. & RASMUSSEN, A. T. (1940). Effects of heating hypothalamus of dogs by diathermy. *J. Neurophysiol.* **3**, 329–338.

HOLMES, R. L., NEWMAN, P. P. & WOLSTENCROFT, J. H. (1958). Location of a heat-sensitive region in the medulla. *J. Physiol.* **142**, 55–56*P*.

LIM, P. K. & GODDINS, F. S. (1955). Control of thermal panting. *Amer. J. Physiol.* **180**, 445–449.

MAGOUN, H. W., HARRISON, F., BROBECK, J. R. & RANSON, S. W. (1938). Activation of heat loss mechanisms by local heating of the brain. *J. Neurophysiol.* **1**, 101–114.

NISBET, W. (1956). An instrument for measuring respiratory rates and volumes. *J. sci. Instrum.* **33**, 154–158.

NISBET, W. (1958). A simple electric respiration counter. *J. Physiol.* **144**, 4–5*P*.

RANSON, S. W. (1940). The Hypothalamus. Chap. XI. Regulation of Body Temperature. *Res. Publ. Ass. nerv. ment. Dis.* **20**, 342–399.

STRÖM, G. (1950). Effect of hypothalamic cooling on cutaneous blood flow in the unanaesthetized dog. *Acta physiol. scand.* **21**, 271–277.

*J. Physiol.* (1961), **155**, *pp.* 86–97
*With 6 text-figures*
*Printed in Great Britain*

# FROG'S SPINAL CORD POTENTIALS GENERATED BY STIMULATION OF CUTANEOUS NERVES

BY MARIA C. BRAVO AND A. FERNANDEZ DE MOLINA
*From the Departamento de Biofisica, Centro de Investigaciones Biologicas, C.S.I.C., Madrid, Spain*

(*Received* 29 *July* 1960)

The potential changes recorded from the dorsal surface of the frog's spinal cord after stimulation of a dorsal root have been analysed by Bonnet & Bremer (1952) in spinal preparations. In 1953 Kolmodin & Skoglund investigated the distribution of the intramedullary potential changes evoked in isolated segments of the frog's cord by dorsal root stimulation; they were able to record typical potential pictures from four different regions of the cross-section. Brookhart & Fadiga (1960) have produced evidence of monosynaptic activation of the motoneurones by both dorsal-root and lateral-column stimulation in isolated frog's cord depressed with pentobarbital; by mapping the potential changes they showed that the post-synaptic responses were confined to a dorsal horn area occupied by interneurones and motoneuronal dendrites, when evoked by dorsal-root stimulation, and to the ventral horn when motoneurones were excited by impulses in the lateral column. Dorsal-surface potentials elicited by stimulation of cutaneous and muscle nerves have been reported in a short account by Marx (1959).

The distribution of potential changes generated in the spinal cord of the cat after stimulation of muscle and cutaneous afferent fibres has been investigated by Eccles, Fatt, Landgren & Winsbury (1954), Coombs, Curtis & Landgren (1956) and Fernandez de Molina & Gray (1957); it was possible to identify in certain areas groups of neurones which responded in a specific manner to activation of a particular group of afferent fibres. Such a study has not yet been made in the spinal cord of the frog: the potential changes recorded by Kolmodin & Skoglund (1953) are complex because of the compound nature of the dorsal root volley. The present investigation was undertaken in order to study the distribution of potential changes evoked in the spinal cord of the frog after selective activation of cutaneous and muscle afferents. It is the purpose of this paper to describe experiments dealing with activation of cutaneous afferents and designed to obtain information about the nature of the

cord's responses and the location of the structures responsible for their generation. A preliminary account of this work has already appeared (Bravo & Fernandez de Molina, 1960*a*).

## METHODS

*Preparations.* The experiments were performed on frogs (*Rana esculenta*). After ether anaesthesia the vertebral canal was opened and the brain stem cut either above the optic lobes or, in a few experiments, at the caudal end of the medulla; the brain above the cut was then destroyed. The spinal cord was exposed from the VI segment down to the caudal tip. Nerves in one limb were prepared for stimulation, usually the dorsolateral pedicular or the lateral crural. The animal was then mounted in a brass frame, vertebral processes being fixed by special clamps. The whole leg with dissected nerves was introduced into a paraffin pool. The white fat of the dura was removed and the arachnoid stripped from the dorsal surface of the VIII, IX and X segments.

*Stimulation.* Stimuli were applied to the nerve through platinum wires from a square-wave stimulator having the output isolated from earth and of low impedance. The duration of the pulses was 0·2–0·4 msec.

*Recording.* Glass capillary electrodes filled with 4 M-NaCl, and with a tip diameter of 1–4 $\mu$ and a resistance of 2–4 M$\Omega$, were used to record the potential changes generated in the cord. The electrolyte solution was connected to the grid of a cathode follower through a chlorided silver wire. The indifferent electrode, a small chlorided silver plate, was attached to the back muscles and the frame was earthed. An RC-coupled differential amplifier with a time constant of 5 sec was employed and the potential changes were displayed on one beam of a double-beam oscilloscope and photographed. All the records shown in this paper were obtained extracellularly with signal negativity of the micro-electrode tip as an upward deflexion. In some experiments the afferent volley was recorded from a filament of the dorsal root and the reflex discharges from the ventral root by means of platinum wires; after suitable amplification the potentials were displayed on the second beam of the oscilloscope.

*Experimental procedure and histological checking.* These have been described in a previous paper (Fernandez de Molina & Gray, 1957). Owing to the small size of the frog's cord no more than two tracks could usually be made in each transverse plane. In some experiments the insertion of the micro-electrode was made through the lateral surface of the cord in order to reach the motoneurones more easily.

## RESULTS

### *Dorsal surface potentials*

Figure 1 shows three records obtained from the dorsal surface of the cord at the level of the IX segment in different experiments, after stimulation of the dorsolateral pedicular nerve with a strength above threshold for polysynaptic reflex discharge. The potential sequence is made up of four deflexions. The first deflexion (*S*) is the action potential of the incoming sensory fibres, consisting of a small positive followed by a large negative deflexion; the positive component was better observed when the pia was opened and the micro-electrode tip placed in the dorsal column or in the dorsal horn, as is the case in record (*c*) on the left in Fig. 6. This initial spike has a latency which corresponds to the time for transmission of the volley in the largest cutaneous fibres.

The deflexions which follow the primary spike we shall call phases of activity (Fernandez de Molina & Gray, 1957). The first phase reaches its peak 1·8–2 msec after the negative peak of the incoming volley and has a total duration of 12–14 msec (Fig. 5*a*). The latency of this phase, counted from the reversal point of the initial positivity of the spike to the onset of the potential (Fig. 6*c*, left column, between arrows) averages 1·2 ± 0·065 msec ($n$ = 21), a time which is in agreement with that reported by Brookhart & Fadiga (1960) for synaptic delay in the frog's spinal cord. The second phase rises from the descending limb of phase I, reaching its peak

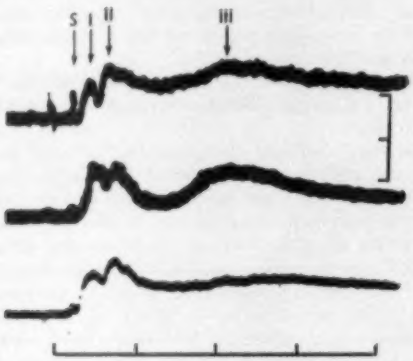



Fig. 1. Records from different experiments to show, *S* the afferent primary spike, and I, II and III the three phases of cord activity. Time marker, 10 msec; voltage calibration in 0·4 mV steps.

3·5–4 msec after the negative peak of the primary spike; the amplitude of this phase at the surface of the cord is usually bigger, in a few experiments equal, but never smaller than that of phase I. Its total duration cannot be determined with accuracy because it becomes obscured by the rising limb of the third phase, although it is of a similar order to that of the phase I, as can be seen when the third phase is abolished by stimulating with a higher frequency (see below). Phase III has a much slower time course, its peak being reached 20–25 msec after the negative peak of the initial spike and its time of decay being about 200–250 msec.

When two cutaneous nerves, the dorsolateral pedicular (P) and lateral crural (C), were maximally stimulated in such a way that both afferent volleys arrived simultaneously at the cord, the three phases of activity showed occlusion. Typical figures in arbitrary units were, for phase I, P = 14, C = 9, P+C = 17; for phase II, P = 17, C = 13, P+C = 20; and for phase III, P = 11, C = 7 and P+C = 12. The occlusion for phase III was almost complete.

*Intramedullary distribution*

When recording at various depths along tracks in a dorsoventral direction a characteristic pattern of distribution of the three phases of activity was observed. Figure 2 illustrates two series of records obtained with different sweep speeds in one of such tracks, passing along the dorsal horn, intermediary region and ventral horn as is shown by the line *A* in Fig. 4. The figure to the left of each record gives the depth in millimetres below the dorsal surface of the cord at which it was obtained. In this

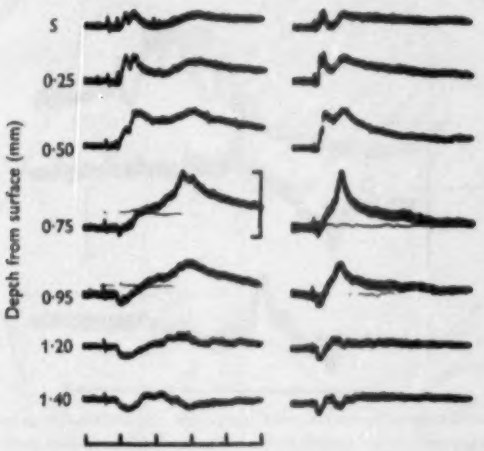



Fig. 2. Series of records obtained with different sweep speeds to show the focal potentials recorded at the surface (*S*) and at the indicated depths (ordinate) along the track shown by line *A* in Fig. 4 after stimulation of the dorsolateral pedicular nerve with a strength 5 times threshold. Time, 10 and 30 msec intervals for the left and right series respectively; voltage calibration 1 mV.

At depth 0·25 mm, phase I maximal; 0·50 mm, phase II maximal, phase I decreasing; 0·75 mm, phase I reversed, phase II considerably decreased and phase III maximal; 0·95 mm, phases I and II reversed, phase III decreased; 1·4 mm, phase III completely reversed.

particular experiment the dorsolateral pedicular nerve was stimulated but the pattern of distribution was essentially the same when the lateral crural or the posterior femoris were stimulated and records obtained at the optimum segmental level.

The incoming volley could be recorded at the dorsum of the cord and dorsal horn, then became smaller and later could no longer be seen (from fourth record downwards). In this experiment phase I reached its maximum amplitude at 0·3 mm, then diminished in size, and at 0·6 mm

reversed to a positive potential of similar time course, which is seen in all the deeper records. Phase II had its maximum more ventral than phase I, at about 0·5 mm, then decreased in amplitude and at 0·8 mm began to reverse to a positive potential which was well developed from the depth of 1 mm downwards. Phase III reached its maximum even more ventrally, at a level (0·8 mm) at which phase I had completey reversed and phase II had begun its reversal. This maximum was situated at the level of the intermediary region. More ventrally it diminished in amplitude and near the level of the motoneurone pool it reversed to a positive potential with a shorter time course.

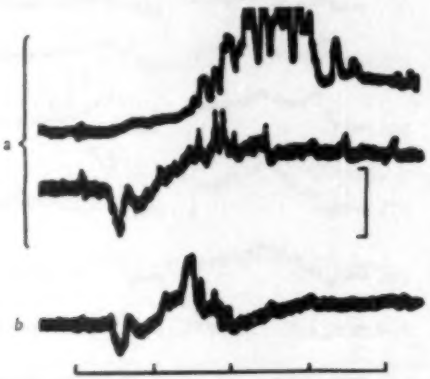



Fig. 3. Motoneurone and ventral-root responses. *a*, simultaneous recording from the IX ventral root (upper trace) and within the motoneuronal pool (lower trace) after stimulation of the dorsolateral pedicular nerve at the level of the lower half of the IX cord segment. *b*, another experiment, record obtained from a position slightly medial to the motoneurones (upper limit of the IX cord segment) after stimulation of the same nerve. In both experiments the micro-electrode was inserted through the lateral surface of the cord. Time marker, 10 msec; voltage calibration applies to all records: *a*, upper trace 500 $\mu$V, lower trace 800 $\mu$V and *b*, 700 $\mu$V.

In the track just described the electrode passed medial to the motoneurone pool. Figure 3 illustrates two records obtained at the level of the motoneurones in two different experiments in which the micro-electrode was inserted through the lateral surface of the cord. When the micro-electrode tip was placed within the motoneuronal pool the record obtained consisted of two initial positivities, which correspond in time course to dorsal negativities (phases I and II), followed by a complex negative wave with some spikes superimposed on it (Fig. 3*a*, lower record). These spikes have a latency of a similar order to that of the reflex discharges recorded from the ventral root (Fig. 3*a*, upper record). Record (*b*) was obtained in

a position slightly medial to the motoneurones and the same sequence of potential changes can be seen; but in this record the ventral horn negativity is interrupted by the development of the third positivity. In a still more medial position this late positivity is recorded without being masked by the ventral negative wave (bottom records in Fig. 2).

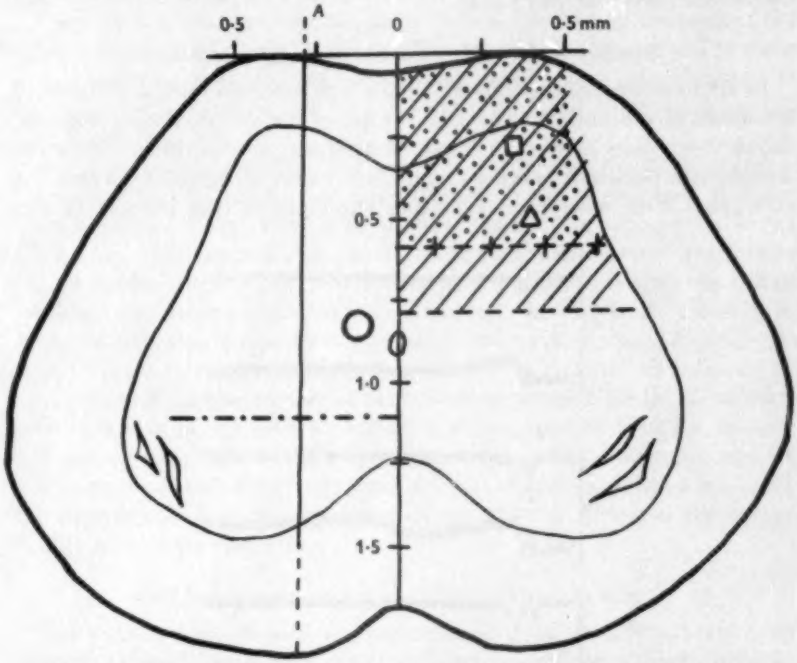



Fig. 4. Distribution of phases of cord potential, all ipsilateral. Right side: dotted region, phase I, □ maximum of phase I; hatching, phase II, △ maximum for phase II; cross-marked and interrupted lines are reversal levels for phases I and II respectively. Left side: ○ maximum for phase III; dash-dot line is the reversal level of this response, which was recorded in the grey substance above this line and in the dorsal column. *A* gives course of the track along which the potentials shown in Fig. 2 were recorded. Lateral columns were not explored.

The distribution of the three phases of activity, as deduced from series of tracks made in different experiments, is shown in Fig. 4; the right side refers to phases I and II (dotted and single-hatched zones respectively) and the left side to phase III. The two first phases have their maxima in the dorsal horn, that of phase I being more dorsal than that of phase II. The lateral positions of these optima could not be determined accurately because usually no more than two tracks could be made in each trans-

verse plane, but both phases tend to be greatest at about 0·4 mm from the middle line, near the line of entrance of the dorsal root. Cross-marked and interrupted lines show the levels of reversal for phases I and II respectively. Phase III has the best defined maximum level and this corresponds to the intermediary region near the ependymal canal; dash-dot line shows the reversal level for this phase.

*Relation of the cord potentials to the afferent volley*

In six experiments the afferent volley was recorded from a filament of the dorsal root simultaneously with the dorsal surface potentials. Figure 5 shows three pairs of records obtained in one such experiment when the dorsolateral pedicular nerve was stimulated with progressively increasing strengths. With a strength 1·2 times threshold (*a*) the phase I is well

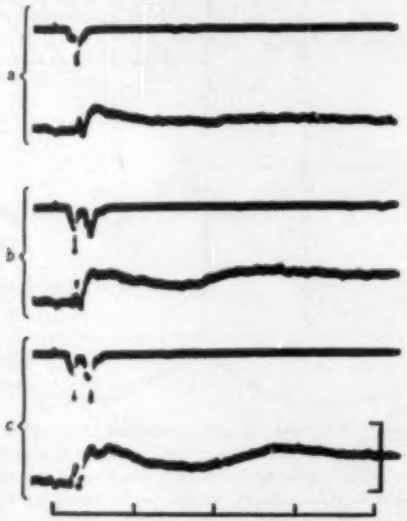



Fig. 5. Stimulus strength. In all records, top beam, afferent volley in a dorsal root filament and bottom beam, cord potentials. *a*, *b* and *c* stimulus strength: 1·2, 2 and 5 times threshold. Cord potentials were recorded from the dorsal surface. Time marker 10 msec; voltage calibration, 1·3 mV for top beam and 0·6 mV for bottom beam.

developed together with a small component of phase III and one spike can be seen in the dorsal-root action potential; the conduction velocity for this group of fibres is in the range of 25–28 m/sec. As the strength of stimulation is increased (2*T*) the second phase rises from the descending limb of the first one, the third phase increases in amplitude and simultaneously

a second spike develops in the action potential recorded from the dorsal root (*b*); this second group of fibres has a conduction velocity of 16–20 m/sec. The record (*c*) was obtained with 5*T* strength; both spikes in the dorsal root record have reached their maximum amplitude and the three phases are completely developed. It is of interest to note that the time between the first afferent spike and the peak of the first phase is of an order similar to that between the second afferent spike and the peak of the second phase. With the intensities and pulse durations employed in these experiments we were not able, even when using higher amplification, to detect a further component in the action potential recorded from the dorsal-root filament.

It might be thought that the second spike in the dorsal-root action potential could be due to repetitive activity in the same group of fibres responsible for the generation of the first spike; this explanation seems unlikely to us for several reasons. In order to obtain a better estimate of the conduction velocity of the pedicular nerve fibres activated, the action potential was recorded both in the sciatic nerve and in the dorsal root; in these experiments the interval between the two spikes was shorter at the level of the sciatic nerve than in the dorsal root, a result which is not to be expected if the same group of fibres were responsible for the generation of both waves in the afferent volley. We have also to consider the low strength of stimulation required to obtain the second afferent spike (in some experiments $1{\cdot}5 \times T$ only), the consistency of its appearance in all the experiments and the regularity of the interval between the spikes during each experiment.

*Effect of repetitive stimulation and barbiturates*

The three phases of cord activity were affected in different ways by repetitive stimulation and by Nembutal (pentobarbitone; Abbott Laboratories) administration. Figure 6 illustrates the effect of various frequencies of stimulation on the amplitude of the phases of the cord potential. Records in the left column were taken when stimulating with a frequency of 10/min and a strength five times threshold; records (*a*) and (*b*) are single traces and record (*c*) was made by superimposing five sweeps. Records in the right column were made as follows: (*a*) is a single trace, the third of a series made with a repetition rate of 15/min; (*b*) and (*c*) are each the second of a series taken when stimulating at frequencies of 20/sec and 50/sec respectively; as the burst of stimuli had a duration of 1 sec there are 20 and 50 traces superimposed on each record respectively. The burst of stimulation was repeated every 3 sec. It can be seen that repetition rates of 15/min suppressed phase III and reduced phases I and II by 10–15%. A frequency of 20/sec reduced both phases I and II by 60%, and these

phases were almost abolished by a repetition rate of 50/sec. The administration of 2 mg of Nembutal (total amount) abolished phase III within 5 min of the injection and reduced the amplitudes of phases I and II by 20%.

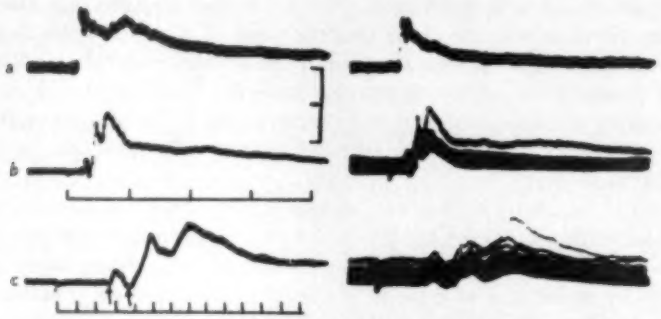



Fig. 6. Effect of repetitive stimulation. Left column: frequency of stimulation, 10/min; *a* and *b* single traces, *c* five superimposed sweeps. Right column: *a*, single trace, frequency of stimulation 15/min; *b*, 20/sec and *c*, 50/sec; in *b* and *c* there are 20 and 50 superimposed sweeps respectively. In *a*, cord potentials were recorded from the dorsal surface; in *b* and *c* (another experiment), the records were obtained at 0·4 mm depth and 0·3 mm from the mid line in the dorsal horn. Time marker under *b*, 10 msec; under *c*, 10 msec for row *a*, and 1 msec for row *c*. Voltage calibration in 450 $\mu$V steps.

## DISCUSSION

Impulses in primary neurones from the skin of the frog can only be recorded in the dorsal half of the grey substance, as has also been shown in the cat (Fernandez de Molina & Gray, 1957). This suggests that most of the cutaneous afferent fibres form synapses with other cells in that part of the cord.

The three phases of activity that follow the primary spike have been shown to be sensitive to repetitive stimulation and to Nembutal, and to exhibit occlusion, a result suggesting that these potential changes can be considered as originating from post-synaptic structures.

The brief latency and time course of phase I as well as its relative resistance to repetitive stimulation and to Nembutal seem to indicate that this potential results from activity in second-order neurones located in the dorsal horn. The afferents that set up this response are the largest from the skin.

The parallel development of phase II and of the second spike in the action potential recorded from the dorsal root supports the view that this second component of the afferent volley activates neurones responsible for the generation of the second phase of cord activity. The time between the

first spike of the afferent volley and the peak of phase I is similar to that between the second afferent spike and the peak of phase II. Phases I and II also respond in a similar way to repetitive stimulation and to Nembutal administration. It has been shown that the maximum amplitudes of phases I and II occur at different levels in the cord, near the dorsal boundary of the grey matter and at the base of the dorsal horn respectively. All these facts are consistent with the inference that phase II originates from activity in second-order neurones located in a more ventral level of the dorsal horn.

The two groups of afferent fibres whose activity is responsible for the generation of phases I and II might be related to the two types of cutaneous receptors ($A_1$ and $A_2$) described by Fessard & Segers (1942) in the skin of the frog.

The third phase had a longer latency and it was more sensitive to repetitive stimulation and to Nembutal than phases I and II. It would seem that this phase represents activity in at least third-order neurones. It could be obtained at stimulus strengths at which phase I was not maximal and continued to increase, with increasing stimulus strength, as the second phase rose from the descending limb of phase I. The maximum amplitude was recorded at the level of the intermediary region. It seems, then, that interneurones located at this level are likely to be activated by impulses generated in interneurones of the dorsal horn, which are themselves activated by impulses in the two groups of primary afferent fibres. However, it should be kept in mind that at this level of the cord a great number of the more dorsal dendrites of motoneurones are also present and we cannot exclude the possibility that activity generated in these dendrites contributed to phase III. This possibility must be considered, since the facilitation of the antidromic response by a cutaneous afferent volley (Bravo & Fernandez de Molina, 1960*b*) begins to appear at the peak of the second phase, a fact which suggests that only one neuronal link exists between cutaneous primary fibres and these dendrites in the shortest pathway to motoneurones.

The sequence of potential changes that we have recorded on the dorsal surface differs from that reported by Marx (1959) in that this author does not mention our phase III. The most probable reason is the extreme sensitiveness of this phase to frequencies of stimulation as low as 12–15/min.

The two first negative potentials recorded in the dorsal horn are paralleled by positive potentials in the more ventral regions of the cord; it could be assumed that these positivities represent source activity for dorsal sinks, because the latencies are identical for both dorsal negative and ventral positive potentials. It would seem then that axons from dorsal

horn interneurones pass into the intermediary region and ventral horn but we have no evidence from which to draw any conclusion about the further course or termination of these axons.

The complex negative potential and superimposed spikes which were recorded at the level of the motoneurones, and which followed the first two positivities, seem to represent asynchronous synaptic potentials and spike potentials generated in the motoneurones. The spike discharges recorded from the ventral root had a latency of a similar order to those recorded in the ventral horn.

The third positivity which can be seen in records obtained at the ventral horn level had a time course somewhat shorter than that of the late negative potential (phase III). Its appearance here depends on the degree of development of the ventral horn negativity. If the assumption is made that phase III is due to activity of both intermediary neurones and dorsal dendrites of motoneurones, this third positivity could then be interpreted as being source activity for these two components. But the possibility cannot be excluded that potentials of an inhibitory nature and of a di-synaptic order take part in the generation of this ventral positivity. Further research is required in order to clear up these problems.

## SUMMARY

1. This investigation is concerned with the potential changes generated in the spinal cord of the frog after stimulation of cutaneous nerves.

2. When recording from the dorsal surface of the cord the primary spike was followed by three phases of potential change.

3. Phase I reached a peak 1·8–2 msec after the primary spike and was elicited by activation of the lowest threshold afferent fibres, having a conduction velocity of 25–28 m/sec. Phase II reached its peak 1·8 msec after the negative peak of a second component of the afferent volley, which had a conduction velocity of 16–20 m/sec. Phase III reached its peak 20–25 msec after the primary spike and developed almost parallel with the two first phases of activity.

4. The three phases were sensitive to repetitive stimulation and to administration of pentobarbital, phase III being the most sensitive. Occlusion between two cutaneous nerves was found for all three phases.

5. The intramedullary distribution of these phases was analysed. Phases I and II had their optimum amplitudes in the dorsal horn, that for phase II being more ventral. Phase III showed its maximum at the level of the intermediary region.

6. Three positive potentials occurred in the ventral horn. At the level of the motoneurones a complex negative wave with superimposed spikes developed after the second positivity; the latency of these spikes was of

the same order as that of the reflex discharge recorded from the ventral root.

7. The nature of these phases of activity has been discussed. It is suggested that phases I and II are due to post-synaptic activity in second-order neurones located at different levels in the dorsal horn. Phase III represents activity in higher-order neurones; the two possible components generating this phase were discussed.

## REFERENCES


Bonnet, V. & Bremer, F. (1952). Les potentiels synaptiques et la transmission nerveuse centrale. *Arch. int. Physiol.* **60**, 33–93.

Bravo, M. C. & Fernandez de Molina, A. (1960*a*). Frog's spinal cord potentials generated by impulses in cutaneous afferent fibres. *J. Physiol.* **152**, 14*P*–15*P*.

Bravo, M. C. & Fernandez de Molina, A. (1960*b*). La activation de motoneuronas en la medula espinal de la rana por aferentes cutaneos. *Rev. esp. Fisiol.* (In the Press.)

Brookhart, J. M. & Fadiga, E. (1960). Potential fields initiated during monosynaptic activation of frog motoneurones. *J. Physiol.* **150**, 633–655.

Coombs, J. S., Curtis, D. R. & Landgren, S. (1956). Spinal cord potentials generated by impulses in muscle and cutaneous afferent fibres. *J. Neurophysiol.* **19**, 452–467.

Eccles, J. C., Fatt, P., Landgren, S. & Winsbury, G. J. (1954). Spinal cord potentials generated by volleys in the large muscle afferents. *J. Physiol.* **125**, 590–606.

Fernandez de Molina, A. & Gray, J. A. B. (1957). Activity in the dorsal spinal grey matter after stimulation of cutaneous nerves. *J. Physiol.* **137**, 126–140.

Fessard, A. & Segers, M. (1942). Dualité des récepteurs tactiles chez la grenouille. *C.R. Soc. Biol., Paris*, **136**, 666–667.

Kolmodin, G. M. & Skoglund, C. R. (1953). Potentials within isolated segments of the frog's spinal cord during reflex activation and changes induced by cholinesterase inhibitors and temperature variations. *Acta physiol. scand.* **29**, Suppl. 106, 503–529.

Marx, Ch. (1959). Actions centrales des influx d'origine cutanée et musculaire chez la grenouille. I. Potentiel de racine ventrale. *C.R. Soc. Biol., Paris*, **153**, 836–840.

*J. Physiol.* (1961), **155**, *pp.* 98–114
*With 6 text-figures*
*Printed in Great Britain*

# NEUROHYPOPHYSIAL HORMONES OF NON-MAMMALIAN VERTEBRATES

BY H. HELLER AND B. T. PICKERING
*From the Department of Pharmacology, University of Bristol*

(*Received* 3 *August* 1960)

Extracts of non-mammalian neurohypophyses, when tested in mammals or on mammalian tissues, produce the same pharmacological effects as mammalian posterior pituitary preparations. Table 1 shows the phyletic distribution of these activities. Relative to their oxytocic, pressor and antidiuretic potencies in mammals, however, non-mammalian extracts have been shown (Heller, 1941*b*; Lazo-Wasem & Weisel, 1952; Sawyer, 1957) to exert a much greater effect on the water balance of frogs. It was therefore suggested (Heller, 1941*b*) that non-mammalian vertebrates secrete a hormone which is different from, but chemically closely related to, oxytocin and vasopressin. Heller & Lederis showed in 1958 that small amounts of neurohypophysial peptides in crude extracts could be separated by paper chromatography and it was therefore decided to re-examine the earlier work on lower vertebrates.

The experiments to be described deal mainly with the chromatographic and pharmacological characterization of the active peptides in pituitary extracts from teleost fishes, amphibians, reptiles and birds. A peptide not demonstrable in mammalian neural lobes was found, and the second part of the paper describes attempts to purify it and to identify its chemical constitution. Preliminary accounts of some of these results have already been given (Pickering & Heller, 1959; Heller & Pickering, 1960).

*Nomenclature.* The term oxytocin is used in reference to the octapeptide characterized by du Vigneaud, Ressler & Trippett (1953) and Tuppy (1953). Konzett & Berde (1959) suggested that other active neurohypophysial peptides and their synthetic analogues should be expressed as derivatives of oxytocin. Thus arginine vasopressin, for example, becomes phe$^{3}$- arg$^{8}$-oxytocin. This nomenclature has been adopted in the present paper.

## METHODS

### *Preparation of extracts*

Pituitary glands were obtained from representatives of the following vertebrate classes: *Marine teleost fishes.* Pollock (*Pollachius virens*). A powder obtained by lyophilizing whole pituitaries was used. The fishes were collected and dissected by Dr Grace Pickford and her

TABLE 1. Phyletic distribution of pharmacological activities in vertebrate neurohypophyses

| Class | Species | Oxytocic | Galactobolic* | Mammalian pressor | Mammalian antidiuretic | Frog water-balance |
|---|---|---|---|---|---|---|
| Elasmobranch fishes | Skate | + (Herring, 1915; Hogben & de Beer, 1925) | + (Herring, 1913) | − (Herring, 1913; Hogben & de Beer, 1925 | + | + |
| | Dogfish | | | | (Heller, 1941*a*) | (Heller, 1941*b*) |
| Teleost fishes | Cod | + (Hogben & de Beer, 1925) | + (Herring, 1913) | + (Herring, 1913) | + (Heller, 1941*a*) | + (Boyd & Dingwall, 1939) |
| Amphibians | Frog | + (Hogben & de Beer, 1925) | Not investigated | + (Herring, 1913) | + (Heller, 1941*a*) | + (Heller, 1941*b*) |
| | Toad | | | (Herring, 1913) | (Jørgensen, 1950) | (Jørgensen, 1950) |
| Reptiles | Tortoise | + (Hogben & de Beer, 1925) | Not investigated | + (Herring, 1913) | + | + |
| | Grass snake | | | (Heller, 1942) | (Heller, 1942) | (Heller, 1942) |
| Birds | Fowl<br>Duck | + (Hogben & de Beer, 1925) | + (Mackenzie, 1911) | + (Herring, 1915) | + | + |
| | Pigeon | | | | (Heller, 1941*a*) | (Heller, 1941*b*) |
| Mammals | All species investigated | + (Dale, 1909) | + (Ott & Scott, 1910) | + (Oliver & Schafer, 1895) | + (von den Velden, 1913) | + (Brunn, 1921) |

+ = present; − = not demonstrated; * = milk ejection.

assistants working under a grant from the U.S. National Science Foundation (NSF G-7262). The pituitaries were deep-frozen and sent to Professor A. E. Wilhelmi who treated them as follows: The glands were thawed in the cold room, transferred to the lyophilizer and cooled to −20° C. After evacuation of the chamber, the material was warmed to a final operating temperature of 23° C. The dry powder was crushed and dispatched by air. 750 g fresh weight of pollock pituitaries gave 122 g dry powder.

*Elasmobranch fishes.* Two species of dogfish (*Squalus acanthias* and *Scyliorhinus caniculus*).

*Fresh-water teleost fishes.* Trout (*Salmo iridens*).

*Amphibians.* Frog (*Rana temporaria*) and toad (*Bufo bufo*).

*Reptiles.* Tortoise (*Testudo graeca*) and grass snake (*Tropidonotus natrix*).

*Birds.* Fowl (*Gallus domesticus*) and pigeon (*Columba palumbus*).

Fresh glands were obtained from all the above vertebrates. The neuro-intermediate lobes were dissected from all but the trout, from which whole pituitary glands were used.

*Acetic acid extracts.* The fresh glands or the dry powder obtained from pollock pituitaries were extracted with 0·25 % (v/v) acetic acid as described by Heller & Lederis (1959).

### *Assay methods*

*Oxytocic assay.* Heller & Lederis's (1959) modification of the pharmacopoeial procedure (British Pharmacopoeia, 1958) was used.

*Vasopressor assay.* Dekanski's (1952) modification of Landgrebe, Macaulay & Waring's (1946) method was used, but dibenyline was injected instead of dibenamine.

*Antidiuretic assay.* The method of Jeffers, Livezey & Austin (1942), employing intravenous injections into ethanol-anaesthetized rats, was used.

*Galactobolic (milk-ejection) assay.* The method of Cross & Harris (1952) and the recording system described by Fitzpatrick (1960) were used.

*Water-balance assay.* The method was essentially that introduced by Heller (1941*b*). Frogs were kept during the night in separate jars each containing water to a depth of about 1 in. (2·5 cm). Next morning the water was changed, the frogs' urinary bladders were emptied, and the animals were weighed to the nearest 0·05 g. Thereafter the bladders were emptied and the frogs weighed at hourly intervals. Injections were given into a ventral lymph sac 3 hr after the first weighing. The experiment was continued until the animals no longer increased in weight. Since Boyd & Brown (1938) had found that the rate of water uptake is influenced by changes in temperature, the experiments were carried out at 18° C in a thermoregulated room.

Responses to hormones were expressed as percentage increase in body weight. The unit of water-balance activity was originally defined (Heller, 1941*b*) as the activity of 0·5 mg International Standard Powder. However, since the vasopressin in the standard powder also exerts some water-balance activity, synthetic oxytocin (Syntocinon; Sandoz) was used as standard. In other words, the unit is now defined as the (frog) water-balance activity exerted by one (rat uterus) unit of Syntocinon.

*Hen uterus (shell gland) assay.* A laying hen was killed by decapitation and the contractions of a strip of the shell gland were recorded. The following suspension fluid was found most suitable (Heller & Lederis, 1960) (g/l.): NaCl, 6·6; KCl, 0·42; $CaCl_2$, 0·01; $NaH_2PO_4$, 0·1; $Na_2HPO_4$, 0·1; $NaHCO_3$, 2·5. The bath solution was maintained at 40° C.

*Standard preparations.* Pitressin (Parke, Davis and Co.); synthetic oxytocin (Syntocinon, Sandoz); phe$^3$-arg$^8$-oxytocin (arginine vasopressin, prepared from ox neurohypophyses by paper chromatography). All these preparations were standardized against the third International Standard Powder.

*Index of discrimination.* The calculation employed was a modification of that suggested by Gaddum (1955). The potencies of two substances were determined in terms of a standard

preparation and the index of discrimination was taken to be the ratio of these two potencies. If the two substances in question are identical, the index of discrimination will be unity whatever assay method is used.

*Chemical methods*

*Paper chromatography.* Both ascending and descending techniques were used. Solvents employed for the development of paper chromatograms were: (*a*) butanol–acetic acid–water (4:1:5 by vol.); (*b*) ethyl acetate–acetic acid–formic acid–water (18:3:1:4 by vol.); (*c*) cellosolve–ammonium sulphate–water (24:20:56 by wt.); (*d*) α-picoline–water (6:4 by vol.). Two staining methods were used. The chromatograms were sprayed with 0·2 g ninhydrin/100 ml. butanol and then dried and heated to reveal the spots, and/or stained according to the method of Reindel & Hoppe (1954) as described by Heller & Lederis (1958). The peptides were eluted with NaCl solution 0·9 g/100 ml. The dried paper was cut into horizontal strips and each of these was again cut into small pieces which were placed in numbered tubes. An appropriate volume of NaCl solution was added to each tube and the contents thoroughly mixed. After filtering, half of the volume initially used was again added; the pieces of paper were finally squeezed over the filter.

*Preliminary purification of pollock pituitary extracts.* The procedure used was essentially that introduced by Acher, Light & du Vigneaud (1958) for the separation of mammalian neurohypophysial peptides. Sodium chloride was added to acetic acid extracts (pH 4·0) of pollock pituitary powder to a final concentration of 25 g/100 ml. The active protein complex precipitated was suspended in a minimal volume of water (about 10 ml.) and transferred to a dialysis bag. The salts, amino acids and free peptides were removed from the precipitate by dialysis against several 200 ml. portions of water for a total period of 3 hr. The precipitate was washed from the bag with, and dissolved in, acetic acid 0·25 % (v/v), and the active peptides were dissociated from the protein complex by the addition of trichloroacetic acid (TCA) to a final concentration of 10 g/100 ml. The precipitate obtained by this treatment was extracted with 0·25 % (v/v) acetic acid and re-precipitated with TCA. This procedure was repeated twice. The combined supernatants were added to Amberlite IR-45 resin (Standard grade) in the acetate form (3 g resin/5 ml. supernatant), and stirred until the pH rose to about 3·9; the resin was then removed by filtration. The filtrate was a solution of the active peptides free of TCA.

*Ion-exchange chromatography.* The partially purified solution of active peptides from pollock pituitaries was added to a 10 × 1 cm column of Amberlite CG-50 (type II) in the hydrogen form. 0·1 M ammonium acetate (pH 5·0) was run through the column until the eluate had a pH of 5·0. The peptides were eluted from the column by a gradient of concentration and pH produced by the gradual introduction of 0·5 M ammonium acetate (pH 7·7) to a 50 ml. mixing chamber (Bock & Ling, 1954) containing the 0·1 M buffer; 2 ml. fractions of the eluate were collected with a Shandon automatic fraction collector.

*Spectrophotometry.* The optical densities of the eluates from the ion-exchange column were measured at 240 mμ in a Unicam S.P. 500 spectrophotometer using 5 mm cells.

*Peptide hydrolysis.* Approximately 1 mg of freeze-dried *highly purified peptide* (see p. 104) was placed in a thick-walled tube and 2 ml. of 6 N-HCl added. The air in the tube was displaced with nitrogen, the tube sealed and incubated for 48 hr at 110° C. The acid was removed from the hydrolysate by successive dilution and evaporation to dryness *in vacuo*.

*Inactivation with sodium thioglycollate.* Vogt's (1953) modification of the procedure of Ames & van Dyke (1951) was used.

## RESULTS

### *Pharmacological activity of crude (acetic acid) extracts of non-mammalian neurohypophysial tissues*

Quantitative estimations of the potency of extracts of non-mammalian neurohypophyses have not been carried out since Mackenzie (1911), Herring (1913), Hogben & de Beer (1925) and Heller (1941*a*, *b*, 1942) established that glandular extracts from all vertebrate classes produce the same qualitative effects as the mammalian posterior pituitary hormones. Oxytocic, pressor, antidiuretic and frog water-balance activities were therefore assayed in simple extracts of pituitary glands from teleost fishes, amphibians, reptiles and birds.

TABLE 2. Mammalian oxytocic and pressor potency of extracts of fresh pituitary glands from non-mammalian vertebrates

| Species | No. of expts. | No. of glands | Mean activity (m-u./gland) Oxytocic (*O*) (isolated rat uterus) | Pressor (*P*) (rat blood pressure) | *P/O* |
|---|---|---|---|---|---|
| Trout | 2 | 36 | 81 | 75 | 0·93 |
| Frog | 6 | 383 | 32 | 13 | 0·25 |
| Toad | 1 | 24 | 45 | 42 | 0·94 |
| Tortoise | 1 | 6 | 58 | 37 | 0·64 |
| Grass snake | 2 | 22 | 365* | 315* | 0·86 |
| Fowl | 2 | 9 | 268 | 245 | 0·92 |
| Pigeon | 1 | 24 | 32 | 28 | 0·88 |

* Per 100 g body weight.

*Extracts of pollock pituitary powder.* Acetic acid extracts of this material were found to have the following mean potencies (m-u./mg dry powder) in five experiments: oxytocic activity, $60\frac{1}{2} \pm 134$; pressor activity $605 \pm 74$; antidiuretic activity, $577 \pm 158$; water-balance activity (one experiment only), 30,700. Referred to the oxytocic potency, the ratio of these activities was therefore 1·0:1·0:0·96:about 50. The ratio antidiuretic activity: water-balance activity agrees well with that of 48 previously (Heller, 1941*b*) obtained in another teleost, the cod. All these activities were abolished by sodium thioglycollate.

*Extracts of fresh glands from other vertebrates.* Pollock pituitaries were the only ones available in quantity as a lyophilized powder, and extracts of freshly dissected glands from other vertebrates were therefore used. Table 2 shows the results of these assays.

### *Paper chromatography of non-mammalian pituitary extracts*

*Extracts of pollock pituitary powder.* When pollock extracts were chromatographed on paper in the system butanol–acetic acid–water,

oxytocic activity could be eluted from two regions. The faster-moving substance was found (7 experiments) between $R_F$ 0·5 and 0·6, i.e. in the same range as oxytocin. The slower-moving oxytocic substance was found at $R_F$ 0·25–0·35 in all experiments, but some oxytocic activity was usually also found at an even lower $R_F$. The pressor and antidiuretic actions of the extracts were mainly associated with the slower-moving oxytocic substance.

In an attempt to discover whether the oxytocic and pressor effects from the lower region of unidimensional chromatograms were due to a single substance, acetic acid extracts of pollock pituitary powder were also subjected to two-dimensional paper chromatography. The paper was first

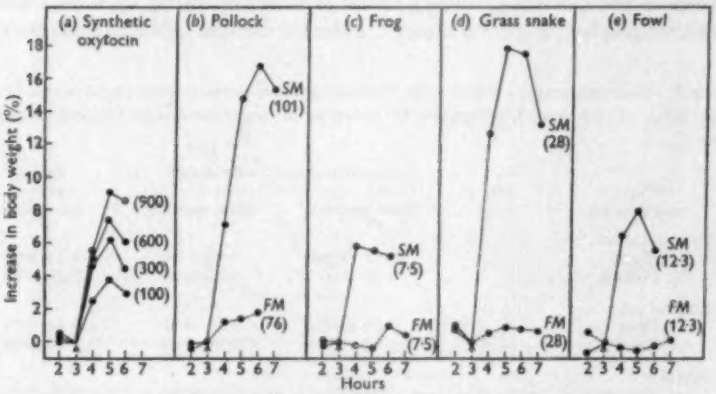



Fig. 1. Comparison of the water-balance potency of the fast-moving (*FM*) and slow-moving (*SM*) oxytocic substances (see text) from non-mammalian pituitary extracts with that of synthetic oxytocin. The results shown are the mean responses of five or six frogs. The figures in parentheses give the doses (m.-u./frog) in terms of (isolated) rat uterus activity. Injections at arrows. Note the disproportion between the oxytocic and the water-balance effect of *SM*.

developed with butanol–acetic acid–water (descending) and then with cellosolve–ammonium sulphate–water (ascending) at right angles to the first development. Oxytocic activity could still be eluted from two regions only. Pressor activity was again associated with the oxytocic substance which moved more slowly in both solvent systems. This suggested that the pollock pituitary extracts did not contain any arginine- or lysine-vasopressin, but indicated the presence of an unknown neurohypophysial peptide with a more pronounced oxytocic potency than the vasopressins. Figure 1*b* shows that the water-balance activity of the pollock gland extracts was mainly due to this unknown peptide.

*Extracts of fresh glands from other vertebrates.* Table 3 shows chromatographic results obtained with extracts of the pituitaries of: a fresh-water

teleost fish (trout), two suborders of anurans (frogs and toads), two orders of reptiles (tortoise and grass snake) and two species of birds (fowl and pigeon). In all these extracts two oxytocic substances were again found which could be eluted from the same regions as in the chromatograms of the pollock pituitary preparations. The pressor and most of the water-balance (Fig. 1) activities were again eluted from the same region as the 'slow-moving' oxytocic substance.

### *Purification of pollock pituitary extracts*

After preliminary purification (see Methods), the two 'oxytocic' substances in the pollock pituitary extracts were separated by ion-exchange chromatography. Figure 2 shows the elution curves. Eluates from the first

TABLE 3. Chromatographic characteristics in the system butanol–acetic acid–water (4:1:5 descending) of the active principles in extracts of non-mammalian neurohypophysial tissue

| Class and species | No. of expts. | $R_F$ of oxytocic substances | | $R_F$ of pressor substance |
|---|---|---|---|---|
| | | 'Fast-moving' | 'Slow-moving' | |
| Teleost fishes | | | | |
| (*a*) Trout | 1 | 0·53–0·69 | 0·26–0·38 | 0·26–0·38 |
| (*b*) Pollock | 7 | 0·50–0·60 | 0·25–0·35 | 0·25–0·35 |
| Amphibians | | | | |
| (*a*) Frog | 3 | 0·50–0·55 | 0·25–0·35 | 0·25–0·35 |
| (*b*) Toad | 1 | 0·55–0·65 | 0·30–0·35 | 0·30–0·35 |
| Reptiles | | | | |
| (*a*) Tortoise | 2 | 0·50–0·65 | 0·25–0·35 | 0·25–0·35 |
| (*b*) Grass snake | 2 | 0·50–0·60 | 0·25–0·35 | 0·25–0·35 |
| Birds | | | | |
| (*a*) Fowl | 2 | 0·50–0·60 | 0·25–0·35 | 0·25–0·35 |
| (*b*) Pigeon | 1 | 0·55–0·60 | 0·30–0·35 | 0·30–0·35 |

active peak (maximum at tube 30), which was in the position of mammalian oxytocin (Acher *et al.* 1958), showed only oxytocic activity. Eluates from the second peak (maximum at tube 80) gave both oxytocic and pressor effects. With the proviso that the contents of every tenth tube only were assayed, the ultra-violet absorption curve resembles the curve of pharmacological activities, in that a single peak was obtained in the same region (tube 80) as for the 'oxytocic-pressor' peptide. There were, however, two peaks in the region of oxytocin, suggesting greater impurity.

The eluates from the region of the second active peak (tubes 75–95) were freeze-dried, dissolved in 2 ml. 0·25 % (v/v) acetic acid and further purified by paper chromatography in butanol–acetic acid–water. The active peptide was eluted with 0·25 % (v/v) acetic acid and lyophilized. This preparation will henceforth be referred to as the '*highly purified pollock peptide*'.

In one experiment the elution gradient was increased by the introduction of 0·75 M ammonium acetate (pH 7·7) to the mixing chamber, after the second active peak had emerged from the column. This procedure (Acher *et al.* 1958) would elute phe$^3$-arg$^8$-oxytocin (arginine vasopressin), but no further activity could be eluted.

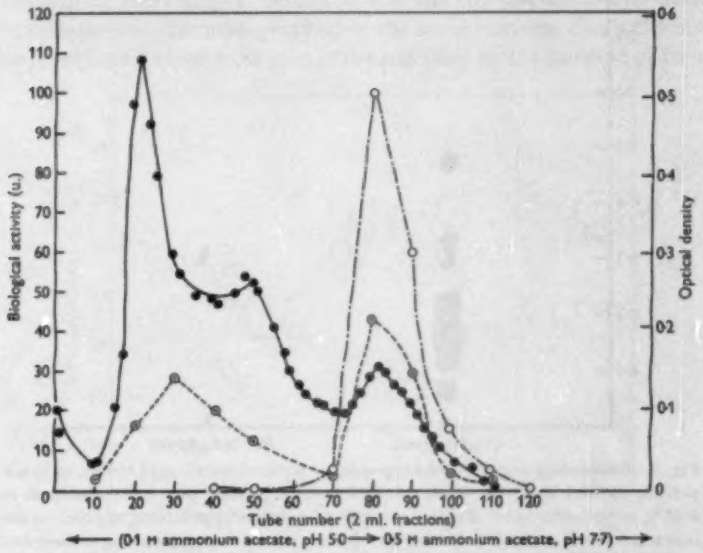



Fig. 2. Chromatography of pollock pituitary extracts on Amberlite CG-50. Elution curves: ●—● optical density at 240 mμ; ⊙···⊙ oxytocic activity; ○----○ pressor activity. For further details see text.

*Identification of the slow-moving peptide in pollock pituitary extracts*

The results so far reported suggested that the non-mammalian pituitary extracts contained a peptide which resembles oxytocin and at least one other peptide which, because of its pronounced oxytocic and frog water-balance activities, differed from phe$^3$-lys$^8$-oxytocin and phe$^3$-arg$^8$-oxytocin, i.e. from lysine- and arginine vasopressin.

While this work was in progress, Munsick, Sawyer & van Dyke (1959) reported that the pharmacological effects produced by extracts of fowl neurohypophyses were compatible with the assumption that such extracts contained oxytocin and arg$^8$-oxytocin (arginine vasotocin), an octapeptide synthesized by Katsoyannis & du Vigneaud (1958). The slow-moving pollock peptide was therefore compared with synthetic arg$^8$-oxytocin, which was kindly provided by Professors van Dyke and du Vigneaud. The two peptides were chromatographed, side by side, in three solvent systems and Fig. 4

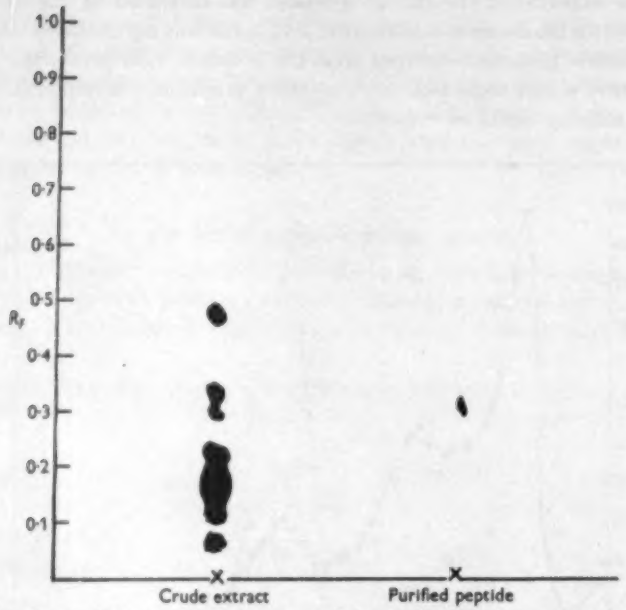



Fig. 3. Descending paper chromatograms, in butanol–acetic acid–water, of crude pollock extract and the *highly purified pollock peptide* (see text) dissolved in 0·25 % (v/v) acetic acid. Pressor activity of volumes of solutions applied: crude extract, 100 m-u.; solution of highly purified peptide, 700 m-u. Staining as described by Heller & Lederis (1958).

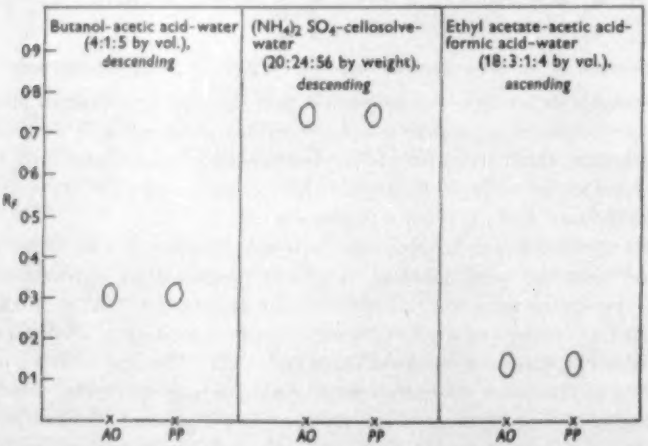



Fig. 4. Synthetic arginine$^8$-oxytocin (*AO*) and the *highly purified pollock peptide* (*PP*) were chromatographed, side by side, in three solvent systems. The figure shows drawings of the results.

shows that the $R_F$ values for the two substances were the same in each solvent.

A sample of the *highly purified pollock peptide* was hydrolysed, and the hydrolysate was subjected to two-dimensional chromatography in (*a*) butanol–acetic acid–water (descending) and (*b*) α-picoline–water (ascending) at right angles. A mixture of the constituent amino acids of arg$^8$-oxytocin was chromatographed in the same manner. Both chromatograms were first stained with ninhydrin and then by the method of Reindel

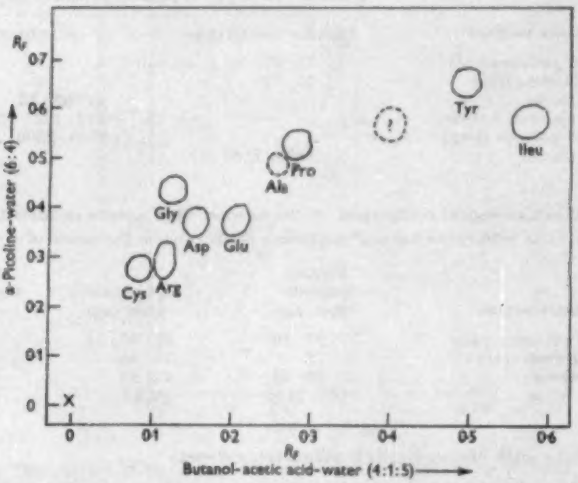



**Fig. 5.** Drawing of a two-dimensional paper chromatogram of a hydrolysate of a sample of the highly purified pollock peptide. The amino acids of arginine$^8$-oxytocin are as follows: (Cys-)$_2$, Arg, Gly, Asp, Glu, Pro, Tyr, Ileu. All these amino acids were found in the hydrolysate, which showed in addition a faint spot corresponding to alanine and another, not stained by ninhydrin, probably due to a peptide fragment.

& Hoppe (1954). The hydrolysate contained all the amino acids of arg$^8$-oxytocin, but two additional 'spots' were found, a faint one in the position of alanine and another, not revealed by ninhydrin, which was probably due to a peptide fragment (Fig. 5). Three such experiments were done.

*Pharmacological potency of the highly purified pollock peptide*

Table 4 shows the potency of our purest preparation of the slow-moving peptide assayed against arginine vasopressin and oxytocin. The highly purified pollock peptide was also compared with synthetic arg$^8$-oxytocin (arginine vasotocin) and the results are shown in Table 5. The pollock peptide could not be differentiated from arg$^8$-oxytocin by any of the assay methods used: the index of discrimination was near unity in every instance.

The similarity of the potencies per microgram of the pollock peptide and synthetic arg$^8$-oxytocin suggests that the former was almost pure. Comparison of the frog water-balance potencies of the two peptide preparations was attempted in only one experiment, but Fig. 6 shows that the potencies were similar.

TABLE 4. Pharmacological potency of the '*highly purified pollock peptide*' (see text). Each value is the result of a (2+2) assay

| Assay method | Potency (m-u./μg) of pollock peptide compared with | |
|---|---|---|
| | Arginine vasopressin | Oxytocin |
| Blood pressure (rat) | 77; 67; 70 | — |
| Antidiuresis (rat) | 70; 72 | — |
| Rat uterus | — | 47; 33; 33 |
| Milk ejection (rabbit) | — | 112; 124; 120 |
| Water-balance (frog) | — | 3500; 2300; 2000 |
| Hen uterus | 1920; 2160 | — |

TABLE 5. Pharmacological comparison of the slow-moving peptide isolated from pollock pituitary extracts with synthetic arg$^8$-oxytocin. Each value is the result of a (2+2) assay

| Assay method | Pollock peptide (m-u./μg) | Arg$^8$-oxytocin (m-u./μg) | Index of discrimination of means |
|---|---|---|---|
| Blood pressure (rat) | 77; 67; 70 | 82; 67; 74 | 0·96 |
| Antidiuresis (rat) | 70; 72 | 76; 84 | 0·89 |
| Rat uterus | 47; 33; 33 | 48; 33 | 1·0 |
| Hen uterus | 1920; 2160 | 2000 | 1·03 |

*Experiments with elasmobranch pituitary extracts*

Perks, Dodd & Dodd (1960) found that neurohypophysial extracts from several species of elasmobranch fishes exerted greater milk-ejection effects than would be expected from their oxytocic activities. They suggested, therefore, that elasmobranchs elaborate an oxytocic principle which differs from oxytocin. They also confirmed the earlier finding (Heller, 1941*a*) that the antidiuretic activity of these extracts is very low.

When elasmobranch pituitary extracts were chromatographed on paper in butanol–acetic acid–water (descending), oxytocic activity could be eluted from two regions. One of these was at the $R_F$ of oxytocin (0·5–0·6) and the other at $R_F$ 0·35–0·45. Four such experiments were done, two with *Squalus acanthias* and two with *Scyliorhinus caniculus* pituitary extracts.

The water-balance activity of dogfish pituitary extract was assayed in two experiments and found to be approximately 40 m-u./gland. The ratio water-balance activity:rat-uterus activity was about 4. These extracts therefore seemed to contain a principle which has more water-balance activity than oxytocin, but is much less potent than arg$^8$-oxytocin.

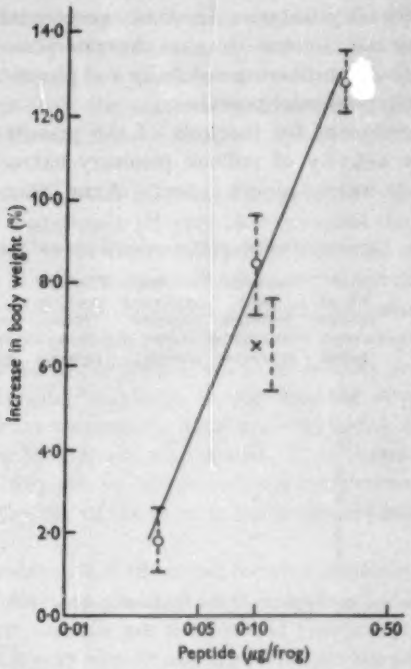



**Fig. 6.** Comparison of the water-balance potency of the highly purified pollock peptide (○) with that of synthetic arginine$^8$-oxytocin (×). Lack of the latter material prevented further experiments. Each point shows the mean and S.E. for 5 frogs. The vertical line indicating S.E. of the results with arg$^8$-oxytocin is displaced to the right for technical reasons.

## DISCUSSION

The results presented suggest that a peptide resembling oxytocin is widely distributed throughout the vertebrate phylum. Chromatographic and pharmacological evidence for its presence was found in two species of elasmobranch fishes, a species of fresh-water and one of marine teleosts, frogs and toads, snakes and tortoises, fowls and pigeons, and in mammals. It has been shown to be oxytocin in the fowl (Acher, Chauvet & Lenci, 1960) but its identity in other vertebrate classes has yet to be established. Neurohypophysial extracts from teleosts, amphibians, reptiles and birds were also shown to contain another active peptide which in a teleost fish, the pollock, has been identified as arginine$^8$-oxytocin (arginine vasotocin). The independent pharmacological observations of Sawyer, Munsick & van Dyke (1959) are in full agreement with these results.

No evidence could be obtained for the presence of arg$^8$-oxytocin in extracts of elasmobranch pituitaries. These extracts contained two oxytocic principles, one with the chromatographic characteristics of oxytocin and the other differing, both chromatographically and pharmacologically, from all identified neurohypophysial peptides.

Arg$^8$-oxytocin accounted for the bulk of the pressor-antidiuretic and frog water-balance activity of pollock pituitary extracts. The pressor-antidiuretic and frog water-balance potency of the other non-mammalian

TABLE 6. Phyletic distribution of neurohypophysial hormones

| Vertebrate class | No. of species investigated | Oxytocin (and peptides resembling oxytocin) | Phe$^3$-arg$^8$-oxytocin (arginine vasopressin) | Phe$^3$-lys$^8$-oxytocin (lysine vasopressin) | Arg$^8$-oxytocin (arginine vasotocin) | Unknown peptide |
|---|---|---|---|---|---|---|
| Mammals | | | | | | |
| (*a*) Domestic pig and hippopotamus* | | + | − | + | − | − |
| (*b*) All others investigated | 13 | + | + | − | − | − |
| Birds | 2 | + | − | − | + | − |
| Reptiles | 3 | + | − | − | + | − |
| Amphibians | 4 | + | − | − | + | − |
| Teleost fishes | 2 | + | − | − | + | − |
| Elasmobranch fishes | 2 | + | − | − | − | + |
| Cyclostomes† | 1 | − | − | − | + | − |

Note: − = absent or not investigated; * see Heller & Lederis (1960); † see Sawyer *et al.* (1959).

TABLE 7. Comparison of the potency of neurohypophysial hormones (m-u./μg), assayed against the third International Standard Powder

| Assay method | Arg$^8$-oxytocin (arginine vasotocin). Mol.wt. = 1036 | Phe$^3$-arg$^8$-oxytocin (arginine vasopressin). Mol.wt. = 1056 | Phe$^3$-lys$^8$-oxytocin (lysine vasopressin). Mol.wt. = 1028 | Oxytocin. Mol.wt. = 993 |
|---|---|---|---|---|
| Rat uterus | 40 | 20–25[1] | 15–20[1] | 360[2], 415[3] |
| Blood pressure (rat) | 71 | 400–450[1] | 270–340[1] | < 10[1], 3·8[3] |
| Antidiuresis (rat) | 71 | 400–450[1] | 110–140[4] | Diuretic[5] |
| Milk ejection (rabbit) | 119 | 70–80[1] | 50–60[1] | 371[3] |
| Hen uterus | 1630 | 320[1] | 29[1] | 29[1] |
| Water-balance (frog) | 2600 | — | — | (360) |
| Urinary bladder (frog) | 7800[2] | 21[3] | < 5[3] | 360[3] |

[1] van Dyke (1959); [2] Sawyer (1960); [3] Berde, Cerletti & Konzett (1960); [4] Thorn (1959); [5] Berde & Cerletti (1956); Brunner, Kuschinsky & Peters (1956); Peters (1959).

pituitary extracts, with the exception of elasmobranchs, was also mainly due to a peptide with the chromatographic and pharmacological characteristics of arg$^8$-oxytocin.

Table 7 compares the potencies per microgram of arg$^8$-oxytocin with those of the mammalian hormones. Since the molecular weights of all

these peptides are much the same (Table 7), the ratio of the activities per microgram virtually equals the ratio per molecule. While many of the results shown are only approximate, the table indicates clearly that, per molecule, arg$^8$-oxytocin has much greater effects on the hen uterus and frog water-balance than the mammalian hormones. In other words, the non-mammalian tissues show a marked specificity for the endogenous peptide.

It is known that the water balance of frogs is more strongly affected by oxytocin than by vasopressin (Heller, 1930) whereas the reverse applies in the toad (Jørgensen, 1950; Ewer, 1951). This led to the suggestion that the neurohypophyses of these anuran suborders contained different hormones. The results now presented, however, support the assumption that both suborders elaborate the same peptide, namely arg$^8$-oxytocin, which may therefore be regarded as the amphibian *water-balance principle* postulated by Heller (1941*b*). Arg$^8$-oxytocin is intermediate structurally between oxytocin and arginine vasopressin (phe$^3$-arg$^8$-oxytocin), differing from each of these hormones by a single amino acid. It is therefore not surprising that amphibians respond to the mammalian hormones, but it may be conjectured that the 'fit' of the peptide molecules to the receptors varies in different anurans.

There is good evidence that the water-balance principle is of physiological importance. The pituitary gland of frogs is depleted of the water-balance principle when the animals are dehydrated (Levinsky & Sawyer, 1953; Jancsó, 1955) and it may also be assumed that the release of arg$^8$-oxytocin aids in maintaining tissue hydration in amphibians by inhibiting water loss through the skin (Boyd & Whyte, 1938), by decreasing urine flow and by increasing the absorption of depot-water from the urinary bladder (Ewer, 1952; Sawyer, 1960). Moreover, arg$^8$-oxytocin is probably also the main antidiuretic hormone of reptiles and birds. The significance of the effects of this peptide on other non-mammalian organs, e.g. on the reptilian and avian oviduct, has yet to be determined.

Our results suggest that, with regard to their neurohypophysial hormones, vertebrates fall into two groups: first, a large group comprising cyclostomes, fresh-water and marine teleosts, amphibians, reptiles and birds (Table 6) which elaborate arg$^8$-oxytocin and a second group, namely elasmobranchs and mammals, which secrete peptides which do not include arg$^8$-oxytocin. Elasmobranchs are unique in that they actively reabsorb urea from their renal tubules, being thus able to utilize a physiological uraemia for osmoregulation. (It will be interesting to see whether this renal tubular function is influenced by the unidentified hormones in their pituitary.) Mammals are characterized by a renal concentrating mechanism which, with the aid of vasopressin, enables them to produce a

hypertonic urine. It is therefore not unlikely that the neurohypophysial hormones produced by each vertebrate class are related to its specific pattern of osmoregulation.

It will be realized that such general conclusions can be only tentative at present. First, because, so far, only a few species within each vertebrate class have been investigated and, secondly, because further work may reveal the occurrence in vertebrate neurohypophyses of hormones additional to those already described.

## SUMMARY

1. Paper chromatography of neurohypophysial extracts from teleost fishes, amphibians, reptiles and birds showed the presence of at least two oxytocic substances. These were designated 'slow-moving' and 'fast-moving' according to their behaviour on paper chromatograms developed in butanol–acetic acid–water.

2. The 'fast-moving' substance resembled mammalian oxytocin in some of its biological and chromatographic characteristics.

3. Pressor and antidiuretic activity was mainly associated with the 'slow-moving' oxytocic substance even after two-dimensional chromatography.

4. The marked frog water-balance effects exerted by non-mammalian pituitary extracts were shown to be due mainly to the 'slow-moving' oxytocic substance.

5. The 'slow-moving' oxytocic substance was isolated by ion-exchange chromatography from pituitary extracts of a teleost fish, the pollock. The purified compound was identified as arginine$^8$-oxytocin (vasotocin) by amino-acid analysis and by chromatographic and pharmacological comparison with the synthetic peptide.

6. Paper chromatography of elasmobranch pituitary extracts suggested that these glands contain a peptide which resembles oxytocin and another oxytocic principle which differs from all other identified neurohypophysial peptides. The water-balance potency of the unidentified peptides was much lower than that of arg$^8$-oxytocin.

Our sincere thanks are due to Dr Grace Pickford and Professor A. E. Wilhelmi who provided large quantities of lyophilized pollock pituitaries. We should also like to express our gratitude to Mr M. Gallop for technical assistance and to the Sir Halley Stewart Foundation for the gift of a spectrophotometer.

## REFERENCES

Acher, R., Chauvet, J. & Lenci, M.-T. (1960). Isolement de l'oxytocine du poulet. *Biochem. biophys. acta*, **11**, 344–345.

Acher, R., Light, A. & du Vigneaud, V. (1958). Purification of oxytocin and vasopressin by way of a protein complex. *J. biol. Chem.* **233**, 116–120.

AMES, R. G. & VAN DYKE, H. B. (1951). Thioglycollate inactivation of posterior pituitary antidiuretic principle as determined in the rat. *Proc. Soc. exp. Biol., N.Y.*, **76**, 576–578.

BERDE, B. & CERLETTI, A. (1956). Über die antidiuretische und diuretische Wirkung von synthetischem Oxytocin im Wasserdiuresversuch an Ratten. *Helv. physiol. acta*, **14**, 129–134.

BERDE, B., CERLETTI, A. & KONZETT, H. (1960). The biological activity of a series of peptides related to oxytocin. In *Oxytocin*, eds. CALDEYRO-BARCIA, R. & HELLER, H. London: Pergamon Press. (In the Press.)

BOCK, R. M. & LING, N. (1954). Devices for gradient elution in chromatography. *Analyt. Chem.* **26**, 1543–1546.

BOYD, E. M. & BROWN, G. M. (1938). Factors affecting the uptake of water by frogs when injected with extracts of posterior hypophysis. *Amer. J. Physiol.* **122**, 191–200.

BOYD, E. M. & DINGWALL, M. (1939). The effect of pituitary (posterior lobe) extract on the body water of fish and reptiles. *J. Physiol.* **95**, 501–507.

BOYD, E. M. & WHYTE, S. W. (1938). The effect of extract of the posterior hypophysis on loss of water by frogs in a dry environment. *Amer. J. Physiol.* **124**, 759–766.

BRITISH PHARMACOPOEIA (1958). London: The Pharmaceutical Press.

BRUNN, F. (1921). Beitrag zur Kenntnis der Wirkung von Hypophysenextrakt auf den Wasserhaushalt des Frosches. *Z. ges. exp. Med.* **25**, 170–175.

BRUNNER, H., KUSCHINSKY, G. & PETERS, G. (1956). Der Einfluss von Oxytocin auf die renale Wasser- und Salzausscheidung der Ratte. *Arch. exp. Path. Pharmak.* **228**, 457–473.

CROSS, B. A. & HARRIS, G. W. (1952). The role of the neurohypophysis in the milk-ejection reflex. *J. Endocrin.* **8**, 148–161.

DALE, H. H. (1909). The action of extracts of the pituitary body. *Biochem. J.* **4**, 427–447.

DEKANSKI, J. (1952). The quantitative assay of vasopressin. *Brit. J. Pharmacol.* **7**, 567–572.

DU VIGNEAUD, V., RESSLER, C. & TRIPPETT, S. (1953). The sequence of the amino acids in oxytocin with a proposal for the structure of oxytocin. *J. biol. Chem.* **205**, 949–957.

EWER, R. F. (1951). The effect of pitressin and pitocin on water balance in *Bufo regularis* Reuss. *J. exp. Biol.* **28**, 374–384.

EWER, R. F. (1952). The effect of pituitrin on fluid distribution in *Bufo regularis* Reuss. *J. exp. Biol.* **29**, 173–177.

FITZPATRICK, R. J. (1960). The estimation of small amounts of oxytocin in blood. In *Oxytocin*, eds. CALDEYRO-BARCIA, R. & HELLER, H. London: Pergamon Press. (In the Press.)

GADDUM, J. H. (1955). *Polypeptides which Stimulate Plain Muscle.* Edinburgh and London: Livingstone.

HELLER, H. (1930). Über die Wirkung der getrennten Hypophysenhinterlappenhormone auf die Wasseraufnahme beim Frosch. *Arch. exp. Path. Pharmak.* **157**, 323–329.

HELLER, H. (1941*a*). The distribution of the pituitary antidiuretic hormone throughout the vertebrate series. *J. Physiol.* **99**, 246–256.

HELLER, H. (1941*b*). Differentiation of an (amphibian) water balance principle from the antidiuretic principle of the posterior pituitary gland. *J. Physiol.* **100**, 125–141.

HELLER, H. (1942). The posterior pituitary principles of a species of reptile (*Tropidonotus natrix*) with some remarks on the comparative physiology of the posterior pituitary gland generally. *J. Physiol.* **101**, 317–326.

HELLER, H. & LEDERIS, K. (1958). Paper chromatography of small amounts of vasopressins and oxytocins. *Nature, Lond.*, **182**, 1231–1232.

HELLER, H. & LEDERIS, K. (1959). Maturation of the hypothalamo-neurohypophysial system. *J. Physiol.* **147**, 299–314.

HELLER, H. & LEDERIS, K. (1960). Posterior pituitary hormones of the hippopotamus. *J. Physiol.* **151**, 47–49*P*.

HELLER, H. & PICKERING, B. T. (1960). Identification of a new neurohypophysial hormone. *J. Physiol.* **152**, 56–57*P*.

HEKRING, P. T. (1913). Further observations upon the comparative anatomy and physiology of the pituitary body. *Quart. J. exp. Physiol.* **6**, 73–108.

HERRING, P. T. (1915). The origin of the active material of the posterior lobe of the pituitary body. *Quart. J. exp. Physiol.* **8**, 245–274.

HOGBEN, L. T. & DE BEER, G. R. (1925). Studies on the pituitary. VI. Localisation and phyletic distribution of active materials. *Quart. J. exp. Physiol.* **15**, 163–176.

JANCSÓ, N. (1955). *Speicherung*, p. 270. Budapest: Akadémia Kiadó.

JEFFERS, W. A., LIVEZEY, M. M. & AUSTIN, J. H. (1942). A method of demonstrating an antidiuretic action of minute amounts of pitressin: statistical analysis of results. *Proc. Soc. exp. Biol., N.Y.*, **50**, 184–188.

JØRGENSEN, C. B (1950). The amphibian water economy, with special regard to the effect of neurohypophysial extracts. *Acta physiol. scand.* **22**, Suppl. 78.

KATSOYANNIS, P. G. & DU VIGNEAUD, V. (1958). Arginine vasotocin, a synthetic analogue of the posterior pituitary hormones containing the ring of oxytocin and the side chain of vasopressin. *J. biol. Chem.* **233**, 1352–1354.

KONZETT H. & BERDE, B. (1959). The biological activity of a new analogue of oxytocin in which the tyrosyl group is replaced by phenylalanyl. *Brit. J. Pharmacol.* **14**, 133–136.

LANDGREBE, F. W., MACAULAY, M. H. I. & WARING, H. (1946). The use of rats for pressor assays of pituitary extracts, with a note on the response to histamine and adrenaline. *Proc. Roy. Soc. Edin.* B, **62**, 202–210.

LAZO-WASEM, E. A. & WEISEL, G. F. (1952). The comparative effects of fish and beef pituitary on the retention of water by frogs. *Biol. Bull., Woods Hole*, **102**, 25–29.

LEVINSKY, N. G. & SAWYER, W. H. (1953). Significance of the neurohypophysis in the regulation of fluid balance in the frog. *Proc. Soc. exp. Biol., N.Y.*, **82**, 272–274.

MACKENZIE, K. (1911). An experimental investigation of the mechanism of milk secretion with special reference to the action of animal extracts. *Quart. J. exp. Physiol.* **4**, 305–330.

MUNSICK, R. A., SAWYER, W. H. & VAN DYKE, H. B. (1959). Hormonal characteristics in avian neurohypophyses. *Fed. Proc.* **18**, 110.

OLIVER, G. & SCHAFER, E. A. (1895). On the physiological action of extracts of pituitary body and certain other glandular organs. *J. Physiol.* **18**, 277–279.

OTT, I. & SCOTT, J. C. (1910). The action of infundibulin upon the mammary secretion. *Proc. Soc. exp. Biol., N.Y.*, **8**, 48.

PERKS, A. M., DODD, M. H. I. & DODD, J. M. (1960). A neurohypophyseal principle in the elasmobranch pituitary. *Nature, Lond.*, **185**, 850–851.

PETERS, G. (1959). Nebennieren und renale Oxytocin-Wirkungen bei der Ratte. *Arch. exp. Path. Pharmak.* **228**, 457–473.

PICKERING, B. T. & HELLER, H. (1959). Chromatographic and biological characteristics of fish and frog neurohypophysial extracts. *Nature, Lond.*, **184**, 1463–1464.

REINDEL, F. & HOPPE, W. (1954). Über eine Färbemethode zum Anfärben von Aminosäuren, Peptiden und Proteinen auf Papierchromatogrammen und Papierelektropherogrammen. *Ber. dtsch. chem. Ges.* **87**, 1103–1107.

SAWYER, W. H. (1957). The antidiuretic action of neurohypophysial hormones in amphibia. In *The Neurophypophysis*, ed. HELLER, H. London: Butterworths.

SAWYER, W. H. (1960). Increased permeability of the bullfrog (*Rana catesbiana*) bladder *in vitro* in response to synthetic oxytocin and arginine vasotocin and to neurohypophysial extracts from non-mammalian vertebrates. *Endocrinology*, **66**, 112–120.

SAWYER, W. H., MUNSICK, R. A. & VAN DYKE, H. B. (1959). Pharmacological evidence for the presence of arginine vasotocin in neurohypophysial extracts from cold-blooded vertebrates. *Nature, Lond.*, **184**, 1464.

THORN, N. A. (1959). Effects of arginine-, lysine- and leucine-vasopressin on urinary osmolarity and rate of urine flow in rats and dogs. *Acta endocr., Copenhagen*, **32**, 134–141.

TUPPY, H. (1953). The amino acid sequence in oxytocin. *Biochim. biophys. acta*, **11**, 449–450.

VAN DYKE, H. B. (1959). Vasopressins. *Proc. XXI int. congr. Physiol.* pp. 61–70.

VOGT, M. (1953). Vasopressor, antidiuretic and oxytocic activities of extracts of the dog's hypothalamus. *Brit. J. Pharmacol.* **8**, 193–196.

VON DEN VELDEN, R. (1913). Die Nierenwirkung von Hypophysenextrakten beim Menschen. *Klin. Wschr.* **50**, 2083–2086.

*J. Physiol.* (1961), **155**, *pp.* 115–133
*With* 10 *text-figures*
*Printed in Great Britain*



# THE TRANSMISSION OF EXCITATION FROM AUTONOMIC NERVE TO SMOOTH MUSCLE

BY G. BURNSTOCK AND MOLLIE E. HOLMAN

*From the Departments of Zoology and Physiology, University of Melbourne, Australia*

(*Received* 8 *August* 1960)

Our approach to the problems of transmission of excitation from autonomic nerve to smooth muscle has been to apply modern electrophysiological techniques to a suitable autonomic nerve–smooth muscle preparation in a manner similar to that described for the skeletal neuromuscular junction (del Castillo & Katz, 1956). In the present experiments the smooth muscle studied was that of the guinea-pig vas deferens. Membrane potentials were recorded intracellularly during repetitive stimulation of the hypogastric nerve.

Rosenblueth (1950) has summarized much of the earlier work in this field, including the results of studies of the electrical activity associated with transmission. He has also dealt with the pharmacology of autonomic junctions and the nature of the chemical transmitters at sympathetic and parasympathetic junctions. The morphology of autonomic nerve endings, as seen from silver and methylene-blue staining, is contradictory and confusing. However, recent studies with the electron microscope, notably those of Caesar, Edwards & Ruska (1957) and Richardson (1958) are at last beginning to clarify the detailed structure of smooth muscle and its innervation.

The properties of the smooth-muscle membrane have been investigated with intracellular electrodes by a number of workers, including Woodbury & McIntyre (1956); Holman (1958); Bülbring, Burnstock & Holman (1958); Daniel & Singh (1958); Jung (1958) and Marshall (1959). The actions of acetylcholine and adrenaline at the membrane level have been described by Bülbring (1957) and Burnstock (1958*a*, *b*, 1960). A preliminary account has been given of some of the work described in this paper (Burnstock & Holman, 1960).

## METHODS

The isolated guinea-pig vas deferens, supplied by the hypogastric nerve, was used in all the experiments. About 1½ cm of the muscle was pinned to a wax square at approximately resting length. The whole was placed in a 3 ml. bath through which modified Krebs's

solution (Holman, 1958) flowed continuously at a rate of about 3 ml./min, at a temperature of 35° C.

The resistance of the capillary micro-electrodes (filled with 3M-KCl) varied from 20 to 50 MΩ. The input stage, amplifiers and camera were conventional.

The electrode used for stimulating the hypogastric nerve consisted of two chlorided silver rings embedded 1 mm apart in insulating 'Araldite'. The portion of the nerve within the electrode was perfused with modified Krebs's solution through fine plastic tubing incorporated between the stimulating rings. The stimulator was of conventional design and was coupled to the nerve by an RF isolation unit.

In some of the experiments, where the chronaxie for direct stimulation was determined, the muscle was mounted in paraffin oil and stimulated with external wick electrodes, as described by Bülbring *et al.* (1958). The same arrangement was used to measure the conduction velocity of the hypogastric nerve.

## RESULTS

### *Response to direct stimulation*

Smooth-muscle cells from the outer longitudinal layer of the guinea-pig vas deferens were penetrated with capillary micro-electrodes. This muscle was never spontaneously active and it was often possible to keep the micro-electrode in a cell for up to 20 min. The cells had resting potentials (inside negative) which varied from 50 to 80 mV (mean, 57 mV; S.E. 6 mV, 50 observations). The resting potential of many cells was not completely steady but fluctuated slowly by ± 10 mV over periods of several minutes. However, 'slow waves' of the type recorded in intestinal muscle (Bülbring *et al.* 1958) were never seen in the vas deferens.

The electrical excitability of the muscle was investigated with external stimulating electrodes. These were applied to the surface of the intact vas deferens or to isolated strips of smooth muscle from the outer longitudinal coat. In both cases the response to a single stimulus, ranging from 2 to 200 msec in duration, usually consisted of a local contraction which could be seen to extend for a few millimetres from the cathode. These local responses occurred whether the preparation was mounted in saline or paraffin oil. The chronaxie under the latter conditions was found to be 10 msec.

Very occasionally the vas deferens responded to a single stimulus with a contraction which extended over several centimetres and was accompanied by an action potential. Figure 1 shows a record from such an experiment. The spike was always preceded by a phase of slow depolarization and was similar in every way to that elicited by stimulating the nerve (see below). It is not possible to say whether these responses arose from direct stimulation of the smooth muscle cells or as a result of stimulation of intramuscular nerve branches. Experiments involving intracellular stimulation of individual cells are in progress and should help to clarify this problem. The only change in membrane potential we have been able

to record during the more common local response was a slow depolarization, similar in time course to that preceding the spike in Fig. 1. In the experiments where the hypogastric nerve was stimulated, contraction was always associated with the initiation of an action potential (see below). This may also have been the case for the local contraction. It is possible that action potentials may have been generated in cells closer to the cathode than the region studied with the intracellular electrode.

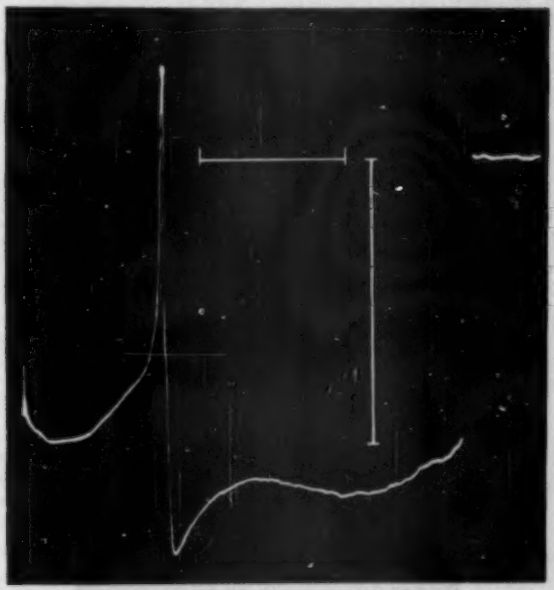


Fig. 1. Intracellular record of the membrane potential of a single smooth-muscle cell of the vas deferens showing the response to stimulation of the muscle with extracellular electrodes (single stimulus, 5 msec duration). Horizontal calibration, 50 msec; vertical calibration, 50 mV. The horizontal trace marks the potential of the electrode outside the cell, i.e. 0 mV.

*Response to nerve stimulation*

Figure 2 illustrates the changes in membrane potential recorded during repeated submaximal stimulation of the hypogastric nerve. In this and subsequent experiments the duration of each stimulus was from 0·5 to 2 msec. The first stimulus gave rise to a slow depolarization of about 5 mV, which had decayed almost to the resting level by the time the second stimulus was given 380 msec later. The response to the second stimulus was greater in amplitude and in rate of rise. Succeeding responses all showed this facilitation. The individual depolarizations summed with

each other until the membrane potential fell to about 35 mV. At this stage an action potential was initiated and the vas deferens contracted vigorously.

We will call the depolarization of the muscle, in response to each stimulus applied to the nerve, a 'Junction Potential'. The term 'End Plate Potential (del Castillo & Katz, 1956) did not seem appropriate, as the morphology of this junction appears to be very different from the skeletal neuromuscular junction (see Discussion). The term 'Post-synaptic Potential' (Eccles, 1957) was considered, but we felt that this should be reserved for junctions between two neurones.

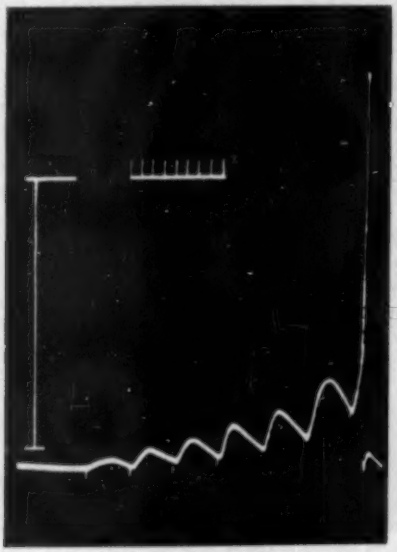


Fig. 2. Intracellular record of the membrane potential of a smooth-muscle cell during repetitive stimulation of the hypogastric nerve (stimulus duration 1 msec). Vertical calibration 50 mV; the horizontal trace = 0 mV; time marker, 100 msec.

The action potentials which are shown in Figs. 1, 2, 4, 7 and 8 were very similar in configuration to those recorded from the guinea-pig taenia coli (Holman, 1958) and the rat uterus (Marshall, 1959); i.e. the rates of rise and decay were similar, and the spike had a symmetrical appearance. The duration at half the total amplitude was about 5 msec. The action potential had a marked after-hyperpolarization which brought the membrane potential back to, or below its resting level. Amplitudes ranged from 57 to 90 mV (mean 68 mV; S.E. 8 mV, 55 observations). The overshoots were up to 20 mV.

The threshold value of membrane potential at which an action potential

was initiated was between 35 and 40 mV. Attempts to arrive at an accurate assessment of this value were complicated by 'tip' potentials which gave rise to an uncertainty as to the absolute value of the resting potential. Moreover, it was not always possible to identify the membrane potential at which the action potential fired, since its 'take-off' was never abrupt, i.e. the foot of the action potential was rounded. However, it was quite obvious that the threshold for firing was far more constant for this preparation than for the spontaneously active guinea-pig taenia coli.

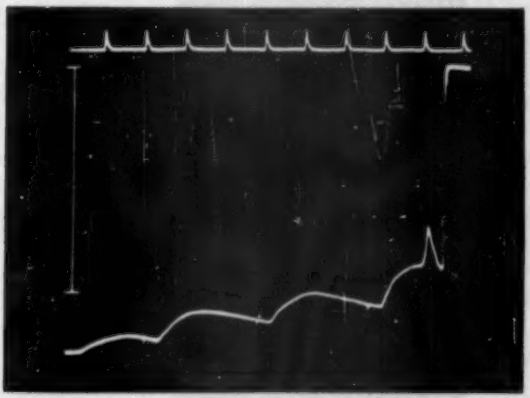


Fig. 3. Membrane potential during repetitive stimulation of the hypogastric nerve; an example of an abortive spike. Vertical calibration, 50 mV; the top of the vertical calibration = 0 mV; time marker, 100 msec.

Occasionally cells were encountered where the threshold for firing appeared to be much lower than $-40$ mV (in one experiment the action potential fired at $-50$ mV). It is tempting to suggest that such action potentials were propagated by current spread from cell to cell and were not initiated by the action of the transmitter on that cell. However, we cannot be certain of this point until more is known regarding the electrical excitability of the smooth-muscle membrane.

Unlike the spontaneously active smooth muscles that have been studied previously, relatively few 'complex' or abortive spikes were recorded from the vas deferens. Figure 3 illustrates one of the few examples encountered. It seems unlikely that this cell could have been injured, since the magnitude of the resting potential and the junction potentials were similar to those of the surrounding cells which gave action potentials of over 80 mV.

*The characteristics of junction potentials*

*Amplitude.* Junction potentials in response to nerve stimulation were recorded in every cell which we impaled. For any one preparation the junction potentials recorded in different cells showed only small differences in amplitude, provided the parameters of stimulation were kept constant.

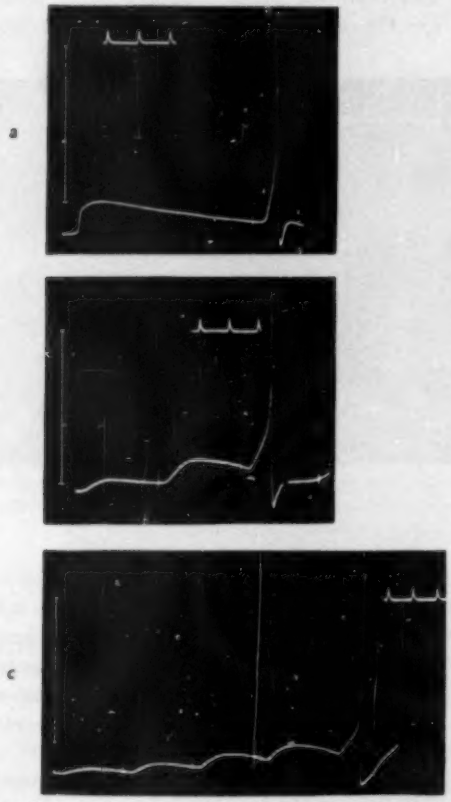



Fig. 4. Three cells from the same preparation showing the effect of decreasing the number of nerve fibres stimulated (for explanation see text). Calibrations, 100 msec and 50 mV, as in Fig. 2.

Thus, if five junction potentials were needed to produce threshold depolarization in one cell, five junction potentials would also be seen to precede the action potential in a cell situated several millimetres away. This finding suggests that the nerve endings must be distributed widely among the smooth-muscle cells.

Individual junction potentials ranged in amplitude from 1 mV up to 20 mV. For rates of stimulation of less than 10/sec, the amplitude of each junction potential depended on

(*a*) the number of preceding transmitter potentials, that is, the degree of facilitation (see below); and

(*b*) the number of nerve fibres stimulated.

The number of nerve fibres stimulated was varied either by altering the strength of stimulation or by allowing some of the fibres in the hypogastric nerve to deteriorate by drying. The records shown in Fig. 4 were taken during an experiment where the stimulus strength was constant, but in the time interval between photographing the different traces the nerve was allowed to deteriorate. Thus, Fig. 4*a* was taken at the beginning of the experiment. The first junction potential was large, having an amplitude of 12 mV. Although 615 msec had elapsed before the next stimulus, there was still some residual depolarization which summed with the second junction potential. At this stage the threshold was reached; thus an action potential was produced by two stimuli. In Fig. 4*b*, taken 1 hr later, the amplitude of the individual junction potentials was greatly reduced. Three stimuli at a higher frequency were required to produce a spike. In Fig. 4*c*, taken 15 min after Fig. 4*b*, seven stimuli were needed. In other experiments similar results were obtained when the strength of the stimulation was reduced. For example, in one preparation three junction potentials were needed to produce a spike when the strength of stimulation was 20 V. Decreasing the strength to 5 V decreased the amplitude of the potentials so that now seven were required to produce threshold depolarization.

*Latency.* Although the amplitude of the junction potentials did not vary greatly over the area of the vas deferens studied in these experiments, there was a marked variation in latency and rate of rise. This finding is illustrated in Fig. 5, which shows the membrane potential changes in two cells with similar resting potentials situated 0·6 mm apart. The junction potentials in the upper record had latencies of more than 50 msec, relatively slow rates of rise and a very gradual 'take-off'. The foot of the junction potentials in the lower record was more clearly defined, their latencies were shorter, and rates of rise faster.

Differences in latency of up to 20 msec were common. In one preparation, which was stimulated submaximally, latencies of 69, 34, 54 and 31 msec were recorded in four cells less than 2 mm apart, although the position of the stimulating electrodes on the nerve was unaltered. The shortest latency recorded was 20 msec. In this experiment the length of nerve between the stimulating electrodes and the intracellular electrode in the muscle was approximately 1 cm. If it is assumed that the nerve

impulse is conducted directly to the muscle fibres with a constant velocity equal to that of the nerve trunk, which was found to be 0·9 m/sec (35° C), a delay of less than 10 msec must have been associated with the transmission process.

*Rates of depolarization and repolarization.* The depolarization or rising phase of the junction potentials showed a relatively abrupt 'take-off' in many cells if stimulus strength was maximal. In other cases the 'take-off' was more gradual and the rising phase was S-shaped. As can be seen in Figs. 2, 3, 4, 5 and 9 the rate of rise varied over a wide range. The time taken to reach half-maximum amplitude was of the order of 50 msec for

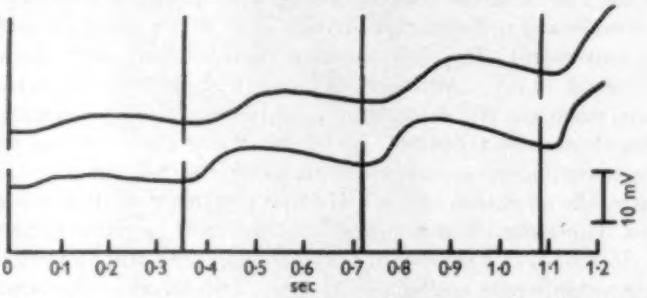



Fig. 5. Tracing of the junction potentials recorded in two cells, having similar resting potentials, situated 0·6 mm apart.

the flattest junction potentials. The larger, faster responses reached half-maximum in 15–20 msec. The time for decay (repolarization) was always longer than the rise time. The rate of decay decreased in time. The time for decay to half-maximum amplitude was of the order of 150 msec.

### *Facilitation*

An increase in the rate of rise and amplitude of the junction potentials in response to successive stimuli was seen in every cell impaled. In Fig. 6 the amplitude of successive junction potentials has been plotted as a function of the time at which each of a train of stimuli was applied to the nerve. In curve *A* the stimulus strength was maximal, and only three stimuli were needed to fire off a spike. The amplitudes of the junction potentials in response to these stimuli increased most dramatically, the amplitude of the third junction potential being more than twice that of the first. Curve *B* shows similar facilitation but under conditions of sub-maximal stimulation, both the stimulus strength and frequency being reduced. Curve *C* shows facilitation occurring although the interval between stimuli was 800 msec. In this case stimulation was sub-threshold for the muscle and it can be seen that the amplitude of the junction potentials reached

a maximum after 8 stimuli. In all our experiments the degree of facilitation was most pronounced for the first 6–8 junction potentials. Facilitation still occurred even when the interval between stimuli was as long as 1 sec.

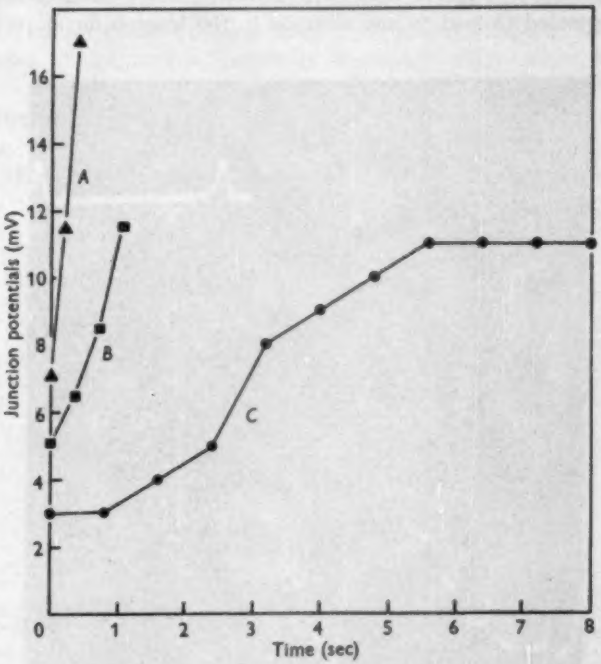



Fig. 6. Amplitudes of successive junction potentials in three different cells, plotted against the time at which successive stimuli were given (for explanation see text).

### *The effect of different frequencies of stimulation*

*High frequencies* (greater than 15/sec). The individual junction potentials in response to stimuli at frequencies greater than 15/sec (at intervals of less than 67 msec) were no longer distinguishable. The over-all rate of depolarization increased with increasing frequencies up to a maximum at about 50/sec. The maximum rate of depolarization recorded in the present experiments was 0·6 V/sec.

Figure 7 shows an example of the membrane potential response to high-frequency stimulation. Following the initiation of a spike after 8 stimuli, the membrane potential returned to its initial level. In this experiment the stimulation was maintained and depolarization began again at a rate similar to that preceding the spike. Thus, for a given stimulus

strength, the rate of depolarization, and hence the frequency at which a series of spikes were initiated, increased with stimulus frequency until a maximum was reached. Since tension depends on firing frequency (Bülbring, 1955; Holman, 1958), frequencies greater than 50/sec would not be expected to lead to any increase in the tension developed by the muscle.

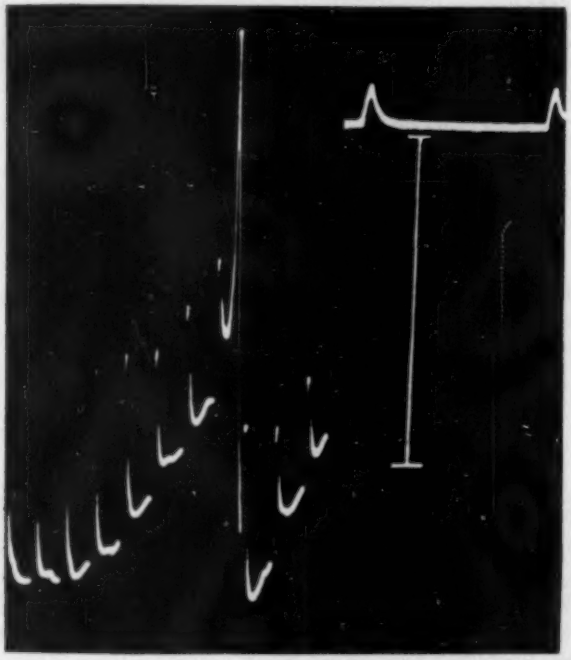


Fig. 7. The membrane potential response to high-frequency stimulation. The trace is interrupted at intervals of 17 msec by the stimulus artifact. Calibrations (50 mV and 100 msec) as in Fig. 2.

*Moderate frequencies* (2–15/sec). Typical responses to stimulation within this range can be seen in Figs. 2, 3 and 4. At the lower end of this range (intervals between stimuli greater than 200 msec) the delay before the *first* spike was initiated was inversely proportional to the frequency of stimulation. Thus, for a given stimulus strength a definite *number* of stimuli were required to produce threshold depolarization. The increased over-all depolarization with successive stimuli was produced more by facilitation than by summation with any depolarization remaining from the previous stimulus. If stimulation was continued after the first spike fewer stimuli were needed to produce the second spike than the first.

This was due to the fact that the junction potentials had by this stage reached their full amplitude and the total depolarization produced by successive stimuli was due to summation.

At the upper end of this range of frequencies summation became an important factor in determining the depolarization produced by the first few stimuli. Thus, as the frequency increased, the number of stimuli needed to fire the first spike was reduced.

*Low frequencies* (less than 2/sec). For any given stimulus strength a minimum frequency of stimulation was required to elicit an action potential. The threshold frequency for stimuli of maximum strength varied

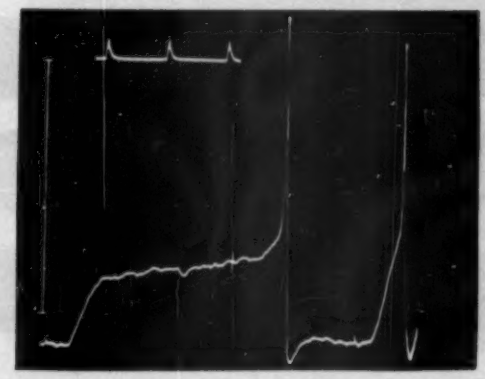


Fig. 8. An example of an action potential in response to the initial stimulus to the hypogastric nerve. The second stimulus was given 430 msec after the first. Calibrations (50 mV and 100 msec) as in Fig. 2.

somewhat from preparation to preparation, probably according to the excitability of the muscle. In most experiments 2 or 3 stimuli were needed and these had to be given at frequencies greater than 1/sec. Only on rare occasions was a single stimulus sufficient. Figure 8 is an example of such a case. The resting potential was 56 mV and the junction potential in response to the first stimulus was 14 mV. This depolarization did not decay. The membrane potential remained at this 'unstable' level for 100 msec before further depolarization occurred and a spike was initiated. A second spike was initiated after a very short delay by the facilitated junction potential in response to the second stimulus.

As the strength of stimulation was reduced, the minimum frequency needed to produce threshold depolarization increased (i.e. the threshold frequency increased). A typical example of sub-threshold stimulation frequency is shown in Fig. 9. Despite normal facilitation of the individual junction potentials, the over-all depolarization was insufficient to reach

threshold (row *A*). It can be seen that the decay of the junction potentials was almost complete before the next junction potential began; the frequency (1·2/sec) was too low to permit any summation. After 15 successive pulses (beginning of row *B*) a fatigue effect became apparent, the junction potentials being reduced in amplitude. Furthermore, an increase in frequency at this stage failed to elicit an action potential. In row *C*,

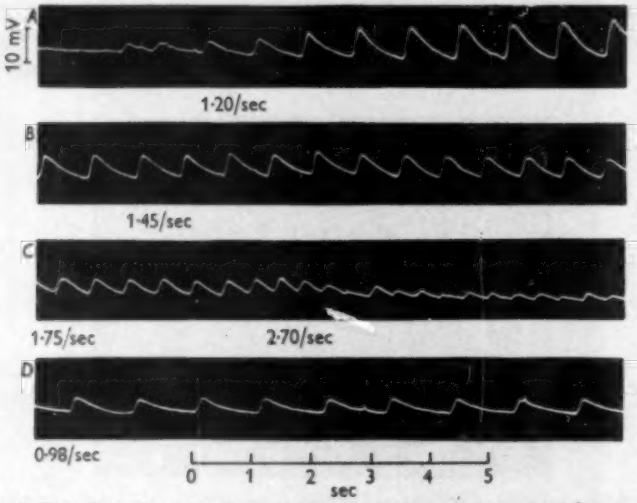



Fig. 9. The effect of continued repetitive stimulation at subthreshold frequencies: records taken at 0 sec (*A*), 11 sec (*B*), 38 sec (*C*), and 60 sec (*D*) after the beginning of stimulation. Frequencies as shown.

38 sec later, the frequency was increased still further. At 2·7/sec the responses to successive stimuli became irregular. Upon returning to a frequency similar to that given initially it can be seen (row *D*) that the junction potentials were reduced to less than half their initial amplitude.

### *Resting discharge*

In the absence of nerve stimulation a spontaneous discharge of what appeared to be 'miniature junction potentials' was recorded from every cell penetrated with the micro-electrode. A typical record of this discharge is shown in Fig. 10. The amplitude of these potentials varied from 15 mV to less than 1 mV, the smallest of them being indistinguishable from the noise level of the apparatus. The smaller potentials were flatter and had a slower rate of rise than the larger ones. Potentials of 6–1 mV appeared about once every 2 sec, but the larger potentials of 6 mV or more had a

lower frequency of about 1/2 min. A detailed analysis of the size, distribution, frequency and time course of these potentials will be dealt with in a later publication.

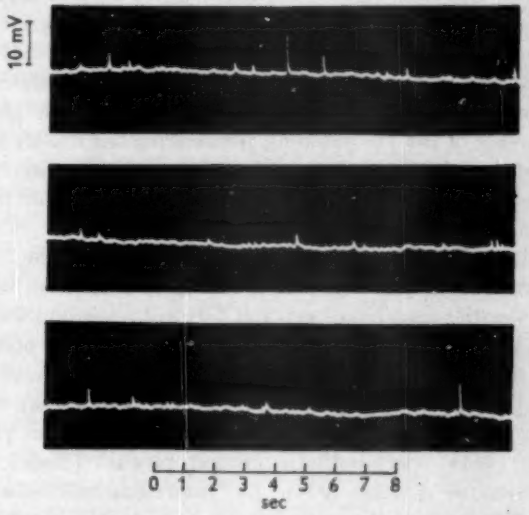



Fig. 10. Continuous record of membrane potential in the absence of nervous stimulation, showing a spontaneous discharge of 'miniature junction potentials'.

## DISCUSSION

We have shown that the mechanism of the transmission of excitation from sympathetic nerve to smooth muscle does not differ fundamentally from transmission at other neuro-effector junctions, stimulation of the effector nerve producing depolarization of the post-junctional membrane. This paper is primarily a description of the main characteristics of this process. A detailed analysis of such features as the time course of transmitter action, facilitation, fatigue, etc., will follow.

We have been unable to find any detailed information on the morphology of the innervation of the vas deferens of the guinea-pig. Langley & Anderson (1896) studied the dog, cat and rabbit and found that stimulation of the sacral nerves or the pelvic nerve near its origin had no effect on the vas deferens. They concluded that this muscle had no nerve supply, either excitatory or inhibitory, from the sacral segments of the cord. Over 90 % of the nerve fibres in the hypogastric nerve of these species are non-myelinated (Langley & Anderson, 1894). Grundfest & Gasser (1938), found that C fibres elevation made up 90 % of the spike area in the cat hypogastric nerve, the conduction velocity of the main peak being 1·6 m/sec.

The conduction velocity of the main elevation of the guinea-pig hypogastric nerve was found to be 0·9 m/sec (35° C). It therefore appears likely that in our experiments only the sympathetic supply to the vas deferens was stimulated and that most of the nerve fibres were post-ganglionic.

In the guinea-pig the hypogastric nerves leave the inferior mesenteric ganglion and run for several centimetres towards the pelvic viscera. Here they fan out into many fine strands which are associated with part of the pelvic plexus. Several branches lead off from this plexus to run along the surface of the vas deferens, penetrating the muscle at intervals. The detailed relationship of the nerve endings themselves with the smooth-muscle cells is no clearer in this preparation than in any other autonomic effector organ.

At this stage it might be profitable to summarize the most widely accepted facts about autonomic nerve endings, rather than to enumerate the various theories that have been put forward regarding their function. It is generally conceded that there exists a fine-mesh plexus of non-myelinated nerves enveloped by a Schwann syncytium and distributed throughout the muscle coats. This plexus has been called variously an 'autonomic ground plexus' (Hillarp, 1959), 'Schwann plasmodium' (Lawrentjew, 1934), 'sympathetic ground plexus' (Boeke, 1949) and 'terminal reticulum' (Stöhr, 1954). An 'interstitial-cell network' is also distributed throughout the muscle. Recent work, including electron micrographs (Caesar *et al.* 1957; Richardson, 1958), has shown that the interstitial-cell network is quite distinct from the autonomic ground plexus and is not formed of Schwann cells. This evidence supports the view that it is formed of connective tissue, probably fibroblasts, rather than of primitive nerve cells. It has been suggested that it forms a link between the autonomic nerve endings and the muscle fibres (Meyling, 1953) but there is little evidence to support this view (Richardson, 1958; Hillarp, 1959).

What happens to the smallest branches of these nerves is still obscure, but has been clarified to some extent recently with electron microscope observations. Richardson (1958) found a few examples of groups of vesiculated nerve fibres lying in an indentation on the surface of a muscle fibre. From his observations on the small intestine of the rabbit he concluded that '...it is most improbable that each muscle fibre receives an ending...'. On the other hand Caesar *et al.* (1957), working with mouse urinary bladder, uterus and gall bladder, concluded '... that each and every muscle cell shows a close relationship to the axon at a well defined locus'. Neither of these authors exclude the possibility that axons passing close to muscle fibres may not possess discrete endings at all, but release transmitter substances at intervals along their length.

There still remains the question of how the peripheral extensions of the post-ganglionic axons form these terminal branches. It seems likely that each post-ganglionic fibre branches many hundreds of times and ramifies extensively throughout the effector organ (Hillarp, 1959). Hillarp has suggested that the terminal branches of several post-ganglionic fibres converge and run within the same strands of the ground plexus, innervating a certain number of cells or cell complexes. There appears to be little evidence to support the view that the autonomic ground plexus is a separate entity, connected synaptically with the post-ganglionic fibres.

The results of our experiments have shed some light on this problem. First, we have shown that junction potentials can be recorded from every cell and therefore the nerve endings must be widely distributed throughout this smooth muscle. Secondly, our experiments suggest that there is a marked degree of convergence of each of the individual post-ganglionic axons on each individual or small group of smooth-muscle cells. Thus, as the number of nerve fibres stimulated was decreased, the amplitude of the junction potentials decreased to a similar extent in all the smooth-muscle cells impaled, at least over areas of up to 1 cm. Lundberg (1955) came to a similar conclusion from his studies on the secretory potentials in cat salivary gland. Further study of the spatial distribution or junction potentials in response to sub-maximal stimulation is required, before the degree of convergence can be finally assessed.

The marked variation in the latency and rate of rise of the junction potentials recorded in the vas deferens suggests that some cells may be activated by a general diffusion of transmitter rather than its liberation at discrete nerve endings on the surface of the muscle. However, the passive electrical properties of this tissue will have to be measured before this suggestion can be verified. The concept of a generalized diffusion of transmitter was first put forward by Rosenblueth (1950) to account for his observation that the responses of autonomic effector organs to changes in *frequency* or *strength* of stimulation were 'quantitatively interchangeable'. We have obtained similar results on the vas deferens. The over-all rate of depolarization in response to nerve stimulation determined the frequency at which action potentials were initiated, and hence the tension developed. (A detailed discussion of the relationship between frequency of firing and tension will be given in a later paper.) This over-all rate of depolarization was dependent on

(*a*) the amplitude of the individual junction potentials and therefore the number of nerve fibres stimulated (stimulus strength), and

(*b*) the frequency of stimulation.

The junction potentials in the vas deferens decayed to half-maximum amplitude in about 150 msec. It seems unlikely that this slow time course

of repolarization could represent the decay of an imposed depolarization in accordance with the passive electrical properties of this tissue. Burnstock, Prosser & Barr (1959) estimated that the membrane resistance of cat intestinal muscle was of the order of 1100 $\Omega$ cm$^2$ and it seems unlikely that the membrane resistance of the vas deferens muscle cells would be abnormally high. It seems more likely that this decay might represent the slow destruction of transmitter. Brown & Gillespie (1957) measured the output of transmitter from cat spleen (isolated but with circulation intact) during stimulation of its sympathetic supply. They estimated that 100 msec was required for destruction of the transmitter liberated by each stimulus to the nerve. Similar studies on cat intestine suggested that the destruction of transmitter might be even slower in this situation (Brown, Davies & Gillespie, 1958). These authors reported that the output of transmitter *per second* reached a maximum at a frequency of 50/sec (cat spleen), remaining constant for higher frequencies of up to 200/sec. This may account for our finding that maximum rates of depolarization in the vas deferens were produced by stimulation at 50/sec, and that increasing the frequency had no effect on the rate at which action potentials were initiated.

The presence of a discharge of 'miniature junction potentials' in the absence of nerve stimulation may help to shed further light on the nature of the nerve endings themselves and the arrangement of the autonomic ground plexus. The finding that they could be recorded in every cell may be taken as further evidence for distributed innervation. Their large amplitude, in relation to the 'miniatures' recorded in other tissues, can probably be accounted for by the relatively small diameter of the smooth-muscle cells. Katz & Thesleff (1957) calculated that the mean amplitude of the 'miniatures' recorded in a neostigmine-treated skeletal muscle fibre of 10 $\mu$ diameter would be as large as 5·6 mV. The maximum diameter of the majority of smooth-muscle fibres in the nuclear region is 6$\mu$, but for most of their length (100–200$\mu$) their diameter is smaller, being of the order of 1–3$\mu$ (Burnstock, Prosser & Kahn, 1960). At present there appears to be no need to suggest that the packets of transmitter are any larger or cause greater depolarization at this junction than at the skeletal neuromuscular junction. The diameter of the vesicles found in autonomic nerve endings in mouse urinary bladder by Caesar, Edwards & Ruska (1957) was 350 Å, which falls within the size range of vesicles described at the skeletal junction (Birks, Huxley & Katz, 1960).

The pharmacology of this junction is under investigation at present by Rand and his co-workers (personal communication). They have found that the response to hypogastric stimulation was unaffected by hexamethonium ($5 \times 10^{-5}$) and atropine ($10^{-6}$) but was blocked by Darenthin ($5 \times 10^{-5}$)

and Priscol ($5 \times 10^{-4}$). These results suggest that this is a typical sympathetic-effector junction and that the transmitter is noradrenaline. In addition, the actions of nicotine, eserine and hemicholinium have been put forward as evidence that an intermediate cholinergic mechanism is involved in sympathetic transmission (see Burn & Rand, 1959). However, regardless of the mechanism of its release it is likely that the junction potentials that have been described in this paper were produced by noradrenaline.

SUMMARY

1. Membrane potential changes of single smooth-muscle cells of the guinea-pig vas deferens were recorded intracellularly during repetitive stimulation of the hypogastric nerve (1–100 pulses/sec).

2. The membrane potential response to each stimulus to the nerve was a depolarization termed a junction potential.

3. Individual junction potentials summed with each other until at a critical over-all depolarization (about −37 mV) a spike was initiated and a contraction occurred.

4. The junction potentials in response to the first 6–8 stimuli showed a progressive increase in amplitude and rate of rise.

5. The vas deferens was never spontaneously active and the muscle cells had resting potentials which varied from 50 to 80 mV.

6. The form of the action potentials resulting from direct stimulation of the vas deferens was identical with that recorded after stimulation via the hypogastric nerve. Amplitudes of action potentials, which were rarely complex, varied from 57 to 90 mV with overshoots of up to 20 mV.

7. Junction potentials were recorded in every cell impaled. Their amplitudes varied little from cell to cell, but varied from 1 to 20 mV within a cell, depending upon the number of nerve fibres stimulated and the degree of facilitation. There was, however, a marked variation in the latency of junction potentials from cell to cell, with differences of up to 40 msec. The shortest latency recorded was 20 msec.

8. The time to reach half-maximum amplitude of the junction potential was about 20 msec; the time for half-decay about 150 msec.

9. The over-all rate of depolarization increased with increasing frequency of stimulation, but reached a maximum of 0·6 V/sec at 50/sec.

10. In the absence of nerve stimulation a spontaneous discharge of 'miniature junction potentials' of up to 15 mV appeared once or twice every 2 sec.

11. Our results have shown that the mechanism of transmission of excitation from sympathetic nerve to smooth muscle is essentially similar to that of transmission at other neuro-effector junctions; stimulation of

the effector nerve producing depolarization of the post-junctional membrane. The results also show that the sympathetic nerve endings must be distributed widely amongst the smooth-muscle cells of the vas deferens. Further, that there is a marked degree of convergence of each of the post-ganglionic axons on each individual or small group of muscle cells.

We would like to thank the National Health and Medical Research Council of Australia for their grant to one of us (M.E.H.). We would also like to thank Professor R. D. Wright for his facilities and his encouragement and Dr D. J. Dewhurst and Mr Ian Pugsley for their help in instrumentation.

## REFERENCES

BIRKS, R., HUXLEY, H. E. & KATZ, B. (1960). The fine structure of the neuromuscular junction of the frog. *J. Physiol.* **150**, 134–144.

BOEKE, J. (1949). The sympathetic endformation, its synaptology, the interstitial cells, the periterminal network, and its bearing on the neurone theory. Discussion and critique. *Acta anat.* **8**, 18–61.

BROWN, G. L., DAVIES, B. N. & GILLESPIE, J. S. (1958). The release of chemical transmitter from the sympathetic nerves of the intestine of the cat. *J. Physiol.* **143**, 41–54.

BROWN, G. L. & GILLESPIE, J. S. (1957). The output of sympathetic transmitter from the spleen of the cat. *J. Physiol.* **138**, 81–102.

BÜLBRING, E. (1955). Correlation between membrane potential, spike discharge and tension in smooth muscle. *J. Physiol.* **128**, 200–221.

BÜLBRING, E. (1957). Changes in configuration of spontaneously discharged spike potentials from smooth muscle of the guinea-pig's taenia coli. The effect of electronic currents and of adrenaline, acetylcholine and histamine. *J. Physiol.* **135**, 412–425.

BÜLBRING, E., BURNSTOCK, G. & HOLMAN, M. E. (1958). Excitation and conduction in the smooth muscle of the isolated taenia coli of the guinea-pig. *J. Physiol.* **142**, 420–437.

BURN, J. H. & RAND, M. J. (1959). Sympathetic post-ganglionic mechanism. *Nature, Lond.*, **184**, 163–165.

BURNSTOCK, G. (1958*a*). The effects of acetylcholine on membrane potential and excitability in the taenia coli of the guinea-pig. *J. Physiol.* **143**, 165–182.

BURNSTOCK, G. (1958*b*). The action of adrenaline on excitability and membrane potential in the taenia coli of the guinea-pig and the effect of DNP on this action and on the action of acetylcholine. *J. Physiol.* **143**, 183–194.

BURNSTOCK, G. (1960). Membrane potentials associated with stimulation of smooth muscle by adrenaline. *Nature, Lond.*, **186**, 727–728.

BURNSTOCK, G. & HOLMAN, M. E. (1960). Autonomic nerve–smooth muscle transmission. *Nature, Lond.*, **187**, 951–952.

BURNSTOCK, G., PROSSER, C. L. & BARR, L. M. (1959). Membrane resistance and conduction in smooth muscle. *Fed. Proc.* **18**, 21.

BURNSTOCK, G., PROSSER, C. L. & KAHN, J. (1960). Comparative studies of conduction in smooth muscle. I. Structural properties. *Amer. J. Physiol.* (In the Press.)

CAESAR, R., EDWARDS, A. & RUSKA, H. (1957). Architecture and nerve supply of mammalian smooth muscle tissue. *J. biophys. biochem. Cyt.* **3**, 867–878.

DANIEL, E. E. & SINGH, H. (1958). The electrical properties of the smooth muscle cell membrane. *Canad. J. Biochem. Physiol.* **36**, 959–975.

DEL CASTILLO, J. & KATZ, B. (1956). Biophysical aspects of neuromuscular transmission; in *Progress in Biophysics* **6**, 122–167. Pergamon Press: London and New York.

ECCLES, J. C. (1957). *The Physiology of Nerve Cells.* London: Oxford University Press.

GRUNDFEST, H. & GASSER, H. S. (1938). Properties of mammalian nerve fibers of slowest conduction. *Amer. J. Physiol.* **123**, 307–318.

HILLARP, N. A. (1959). The construction and functional organization of the autonomic innervation apparatus. *Acta physiol. scand.* **46**, Suppl. 157, 1–38.

HOLMAN, M. E. (1958). Membrane potentials recorded with high resistance micro-electrodes; and the effects of changes in ionic environment on the electrical and mechanical activity of the smooth muscle of the taenia coli of the guinea-pig. *J. Physiol.* **141**, 464–488.

JUNG, H. (1958). Über Membran- und Aktionspotentiale der Uteruseinzelfaser in der Schwangerschaft und unter der Geburt. *Pflüg. Arch. ges. Physiol.* **268**, 60.

KATZ, B. & THESLEFF, S. (1957). On the factors which determine the amplitude of the 'miniature end-plate potential'. *J. Physiol.* **137**, 267–278.

LANGLEY, J. N. & ANDERSON, H. K. (1894). The constituents of the hypogastric nerves. *J. Physiol.* **17**, 177–191.

LANGLEY, J. N. & ANDERSON, H. K. (1896). The innervation of the pelvic and adjoining viscera. *J. Physiol.* **19**, 71–139.

LAWRENTJEW, B. J. (1934). Einige Bemerkungen über Fortschritte und Aufgaben der Erforschung des autonomen Nervensystems. *Z. mickr.-anat. Forsch.* **36**, 651–659.

LUNDBERG, A. (1955). The electrophysiology of the submaxillary gland of the cat. *Acta physiol. scand.* **35**, 1–25.

MARSHALL, J. M. (1959). Effects of estrogen and progesterone on single uterine muscle fibres in the rat. *Amer. J. Physiol.* **197**, 935–942.

MEYLING, H. A. (1953). Structure and significance of the peripheral extension of the autonomic nervous system. *J. comp. Neurol.* **99**, 495–535.

RICHARDSON, K. C. (1958). Electronmicroscopic observations on Auerbach's plexus in the rabbit, with special reference to the problem of smooth muscle innervation. *Amer. J. Anat.* **103**, 99–136.

ROSENBLUETH, A. (1950). *The Transmission of Nerve Impulses at Neuroeffector Junctions and Peripheral Synapses.* New York: The Technology Press of Massachusetts Institute of Technology and John Wiley and Sons, Inc.

STÖHR, P. JR. (1954). Zusammenfassende Ergebnisse über die Endigungsweise des vegetativen Nervensystems. *Acta neuroveg.* **10**, 21–109.

WOODBURY, J. W. & MCINTYRE, D. M. (1956). Transmembrane action potentials from pregnant uterus. *Amer. J. Physiol.* **187**, 338–340.

*J. Physiol.* (1961), **155**, *pp.* 134–147
*With 9 text-figures*
*Printed in Great Britain*

# THE ELECTROMAGNETIC MEASUREMENT OF THE FLOW OF DIGESTA THROUGH THE DUODENUM OF THE GOAT AND THE SHEEP

BY A. G. SINGLETON

*From the Physiological Laboratory, University of Liverpool*

(*Received* 17 *August* 1960)

The quantitative aspects of digestion are of particular importance in ruminants, in which food is subjected to microbial fermentation in the reticulo-rumenal sac before being exposed to gastric and intestinal digestion lower down the alimentary tract. An assessment of the nutritional importance of microbial activity, which includes both break-down and synthesis of food material, requires as a basic measurement the quantity of material leaving the stomach over a suitable period of time.

Phillipson (1952) measured directly the volume of digesta leaving a duodenal cannula. In some of his experiments the duodenum was blocked by an inflated balloon, which reduced the amount of flow; in others, the material flowing out was not returned to the duodenum, and this increased the amount of flow. He suggested that the values obtained by these two methods represented the lower and upper limits, respectively, of the range of true values, which would probably be between 350 and 800 ml./hr.

More recently Hogan & Phillipson (1960) have published the results of experiments performed on sheep with re-entrant duodenal fistulae. Flow through the duodenum was measured by collecting material from the cannula nearest to the stomach and then returning it to the duodenum at short intervals. By this method a mean flow rate of 360 ml./hr was obtained.

In the experiments to be described the flow of digesta through the duodenum has been recorded and measured by a method which avoids the removal of material from the duodenum, or the application of any stimulus known to affect the rate of emptying of the stomach. It is based on the method of recording blood flow in unopened vessels described by Wetterer (1938) and Katz & Kolin (1938) and more recently by Richards & Williams (1953), which uses the principle of electromagnetic induction.

## METHODS

*Animals.* Seven cross-bred goats and four Clun Forest cross-bred sheep were used. They were brought from pasture to the animal house and accustomed to indoor feeding for 2–3 weeks before operation. They were fed twice daily, at 9.0 a.m. and 4.30 p.m., the goats receiving good quality hay and concentrates. The sheep received hay only, because concentrates tended to produce blockage of the cannulae. The animals were kept in stands which were designed both to limit movement and thereby prevent damage to the cannulae and to make possible the measurement of food intake and faeces output.

*Operative procedure.* Anaesthesia was induced by pentobarbitone sodium I.V. and was then maintained by cyclopropane and oxygen in closed circuit after tracheal intubation. The duodenum was exposed and, after ligation of the mesenteric vessels, transected about 2 in. (5 cm) beyond the pylorus. A Perspex cannula of $\frac{3}{8}$ in. (9 mm) internal diameter and about 2 in. in length was fixed in each open end. The cannulae were brought out through stab wounds in the abdominal wall, and joined by polythene tubing and Perspex right-angle bends. It was found that by inserting the cannulae in the open ends of the duodenum larger cannulae could be employed than if the ends of the duodenum were closed and the antimesenteric border cannulated. A successful preparation remains useful for 3–6 months, occasionally longer. After recovery from the operation, food intake and faeces output return to the pre-operative levels, and the animal maintains or gains body weight.

*Apparatus.* The principle of the recording method is stated by Richards & Williams (1953) as follows: 'When a conductor of length $l$ cm moves with a velocity of $v$ cm/sec through a magnetic field of $H$ gauss, an e.m.f. is induced such that $e = Hvl\ 10^{-8}$'. In this application, duodenal contents are the moving conductor, and if they flow through a rigid tube of uniform cross-sectional area the diameter of that tube is equivalent to the length of the conductor. The velocity will be directly proportional to the volume flow in unit time. If the voltage change is recorded on moving paper and if the over-all calibration curve is linear, then the areas between the voltage curve and base line will be proportional to the volume of duodenal contents flowing through the tube in unit time.

The electro-magnetic flowmeter consists of the electrode-holding block, non-polarizable electrodes, a large permanent magnet and electrical recording equipment. The electrode holder is formed from a block of Perspex, shaped so that it can be fitted between the pole-pieces of the magnet and locked in position by a Perspex nut on a brass screw. It includes a central tube through which the duodenal contents pass, and smaller holes which are a close fit for the electrodes and which communicate with the central tube in the correct plane by narrow drilled channels which are filled with agar-saline jelly. It is important that no movement of the electrodes relative to the magnet should be allowed (Fig. 1). The electrodes are miniature calomel half-cells similar to those described by Richards & Williams (1953).

The permament magnet is made of Alcomax III (Messrs Jessop Sheffield) and has mild-steel pole pieces separated by a $\frac{1}{2}$ in. (12 mm) gap, the field strength across which is 10,000 gauss. It rests on a wooden platform suspended from a stand by ropes passing over pulleys and is counter-balanced by lead weights so as to allow adjustment in height for different sizes of animal.

The recording apparatus consists of a push-pull DC pre-amplifier and oscilloscope with moving film camera.

*Experimental.* Recordings are made with the animal standing, lightly restrained between two Pavlov stands which prevent lateral movements. The duodenal cannulae are connected to each end of the central tube of the electrode holder by an 8 in. (20 cm) length of polythene tubing, the flexibility of which allows some movement of the animal without movement of the magnet (Fig. 2). The animals are well accustomed to handling before operation and quickly become used to the laboratory, so that continuous recordings can be made for periods of up to 3 hr.

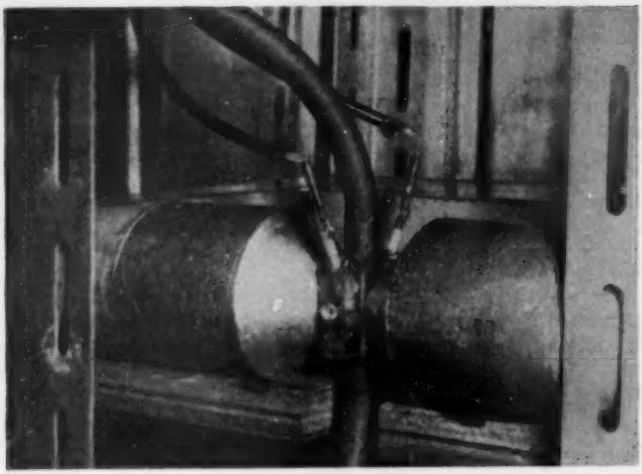


Fig. 1. The permanent magnet on its counter-balanced wooden platform. The electrodes in the Perspex block are rigidly fixed between the pole pieces, and the polythene tubing connects with the central tube of the electrode holder.

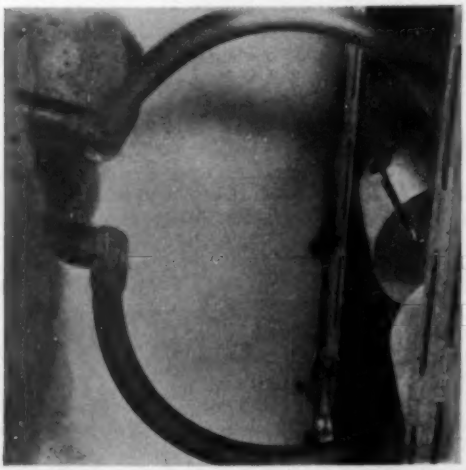


Fig. 2. The flexible connexion between the duodenal cannulae and the electrode holder, which allows some movement by the animal.

The areas between velocity tracing and base line are measured by planimeter, and the relationship between area and volume is established by direct calibration, which has been done by two methods. In the first, measured volumes of sodium chloride solution or duodenal contents are allowed to pass through the Perspex tube of the electrode holder; in the second, digesta from the lower duodenal cannula are allowed to pass through the flowmeter for short periods, but instead of returning them to the upper cannula they are collected and measured.

By the first method calibration is precise. The curves obtained are regular, and the areas easy to measure by planimeter. The second method produces a tracing similar to those obtained during actual experiments, except that antiperistaltic flow is prevented. The areas to be measured are smaller and less regular and include some caused by oscillation of the column of fluid due to movements of the animal, so that the error involved in planimeter measurement is greater.

## RESULTS

### *Calibration*

The passage through the flowmeter of measured quantities of sodium chloride solutions or abomasal contents produces results similar to those described by Richards & Williams (1953) in that, when area is plotted against volume, there is a straight-line relationship, the line passing through the origin. The results of calibration with NaCl solution 0·9 g/100 ml. and duodenal contents are shown in Fig. 3, the line being a regression of area on volume. A comparison of means of the areas obtained with saline and duodenal contents shows no significant difference between the two ($P > 0·4$). The results of calibration on the animal are shown in Table 1. By this method all experimental error of measurement is included.

### *Pattern of flow through the duodenum*

The tracings (Figs. 4 and 5) confirm Phillipson's observation (1952) that flow occurs mainly in gushes, separated by intervals of different lengths, but also show that flow occurs in both directions, a flow of material from the stomach being usually, but not invariably, followed by a smaller return flow, represented by the area below the base line. Small spurts of retrograde flow also occur independently of the main aboral gushes (Fig. 5).

The tracings are primarily a record of velocity change. In any one animal differences occur in the form of individual flows, the rates of acceleration and deceleration and the peak velocity and duration, producing a considerable range of variation in the volume ejected at each gush. The highest peak velocities observed in goats have been of the order of 18 cm/sec but more usually lie within the range 7–11 cm/sec. In sheep the velocities tend to be lower, within the range 4–9 cm/sec, but occasionally as high at 12 cm/sec.

As well as this variation in the volume of individual flows, there is also variation in frequency, and in the size and incidence of the return flows.

The amount of to-and-fro movement is much greater in goats than in sheep, as can be seen from the tracings in Figs. 4 and 5, and from the histograms (Figs. 7 and 8). Feeding has no regular effect on the pattern of flow in animals fed by the normal routine. If an animal is deprived of food for 24 hr the flow is very much reduced and a subsequent feed is immediately followed by an increased rate of flow. Although rumination can occur without any obvious regularity of the duodenal flow it is often accompanied by a flow pattern in which the gushes occur in groups of two to six, separated by short intervals (Fig. 6).

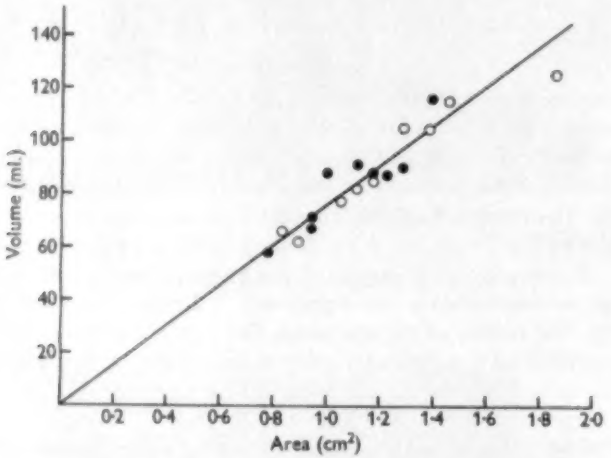



Fig. 3. Calibration of the flowmeter by passing measured quantities of saline (○) and duodenal contents (●) through it. The line is a regression of volume on area.

TABLE 1. Calibration on the animal

| Animal no. | Duration of expt. (min) | Volume collected (ml.) | Area measured ($cm^2$) | Vol./$cm^2$ |
|---|---|---|---|---|
| 31 | 10 | 130 | 1·183 | 110 |
| 31 | 14 | 100 | 0·833 | 120 |
| 31 | 10 | 245 | 2·150 | 114 |
| 31 | 9 | 270 | 2·266 | 119 |
| 31 | 12 | 325 | 2·75 | 118 |
| 61 | 7 | 100 | 0·866 | 115 |
| 52 | 10 | 425 | 3·600 | 118 |

Mean vol./$cm^2$ = 116·3 ± S.E. 1·32.

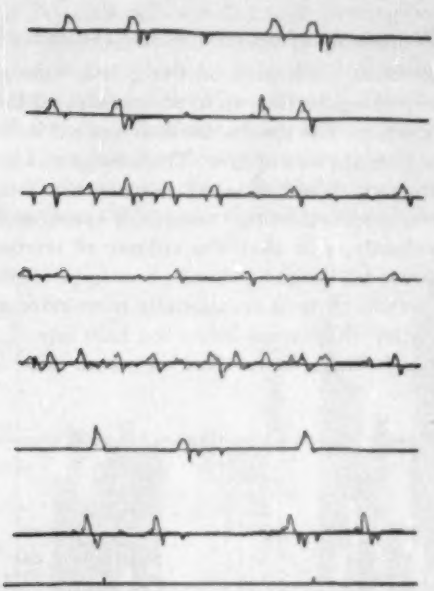


Fig. 4. Examples of flow records from goats, illustrating the variation which occurs. The areas above the base line represent aboral flow, those below retrograde flow. Time marker, minutes.

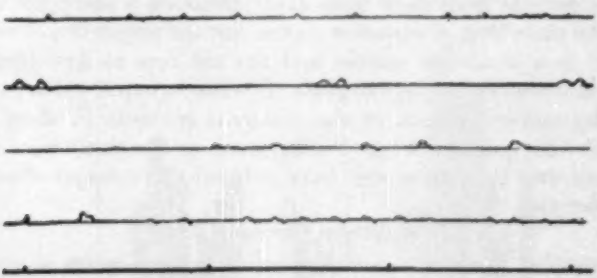


Fig. 5. Representative tracings from sheep. The aboral flows tend to be smaller than in goats, and the quantity of retrograde flow much less. Time marker, minutes.

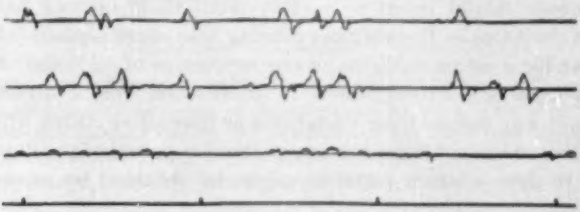


Fig. 6. Examples of a flow pattern often seen during rumination, in which gushes occur in groups separated by intervals. Time marker, minutes.

*The quantity of material leaving the stomach*

The first experiments, performed on five goats, consisted of relatively short periods of recording, lasting up to 80 min and all between the hours of 9.0 a.m. and 10.0 p.m. The results are summarized in Table 2 and show the extent of variation in rates of flow. The histograms in Fig. 7 show the short-term variation which occurs between successive 5-min periods. It is apparent from these results that the volumes of aboral and retrograde flow can vary independently, and that the volume of retrograde flow has a considerable influence on the net volume of material leaving the stomach. Over such short periods there is occasionally more retrograde than aboral flow, as shown by the white areas below the base line. Intervals of up to 15 min during which there is no flow in either direction occur quite commonly.

TABLE 2. Results of short experiments on five goats expressed as flow

| No. of expts. | Goat no. | Wt. (kg) | Mean aboral flow (ml./hr) | Mean retrograde flow (ml./hr) | Mean net aboral flow (ml./hr) | Range | S.D. | Coef. of variation |
|---|---|---|---|---|---|---|---|---|
| 6 | 13 | 35 | 868 | 475 | 392 | 117–684 | 116 | 42 |
| 23 | 31 | 41 | 2037 | 1143 | 893 | 174–1925 | 511 | 57 |
| 8 | 21 | 33 | 863 | 279 | 584 | 191–858 | 255 | 44 |
| 22 | 41 | 34 | 2839 | 1520 | 1319 | 282–2025 | 483 | 37 |
| 7 | 23 | 25 | 1251 | 419 | 803 | 307–1180 | 343 | 43 |

Similar periods from short-term experiments on a sheep are shown in Fig. 8. The same kind of variation occurs, but the proportion of retrograde to aboral flow is always smaller and the net rate of flow through the duodenum is always less than in goats. This can be explained in part by the higher dry-matter content of the duodenal contents in sheep (5–6 %) compared with goats (3–4 %). Furthermore, as the sheep were receiving no concentrates this might well have reduced the volumes of saliva and gastric secretion.

*The volume flowing in 24 hr*

The practical limit for a period of continuous recording is about 3 hr. After this length of time some animals became restless and, as they must remain standing during the whole recording period, it was considered that longer periods would interfere unduly with their normal habits. The amount of variation in the volumes flowing over short periods (about 1 hr) is too great for a mean value to be representative of all hours of the day, and an estimate of the total flow in 24 hr obtained from it would not take into account any longer-term variations or diurnal rhythms which might occur. If the volume of flow over any particular period of the day is similar from day to day, a better estimate might be obtained by recording each

3-hr period of the day on a different day and covering each of the 24 hr in as short a period as practicable. An examination of the results of the short experiments on goats indicated that the hourly flow rates for the morning were of the same order, and different from those in the afternoon (Table 3). These experiments were not planned to show this point, and begin and end at different times of day, but they do suggest a certain degree of consistency at about the same time of each day. It might be expected that longer recording periods such as 3 hr beginning and ending at the same time each

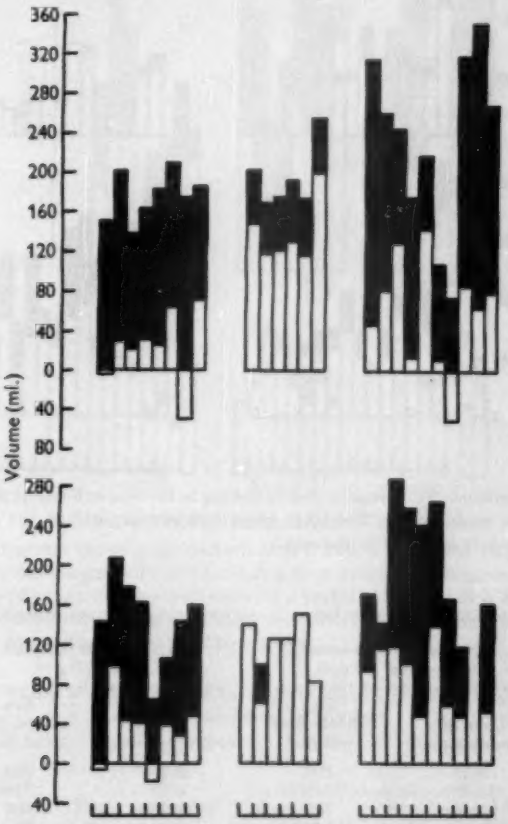



Fig. 7. Quantities of duodenal contents flowing backwards and forwards in successive 5-min periods in six experiments on the same goat. The total height of each block represents the quantity leaving the stomach and the black area denotes the proportion returned as retrograde flow. The white areas thus represent net aboral flow: occasionally, over these short periods, this quantity is negative.

day would show less variation, and this is confirmed by the experiments on sheep 929 (Table 5).

Six 24-hr estimates were made on this basis, two on one goat, and one each on another goat and on three sheep. The results are summarized in the histograms (Fig. 9) and in Table 4. From the histograms it can be seen that the goats show a regular tendency to a period of reduced flow in the

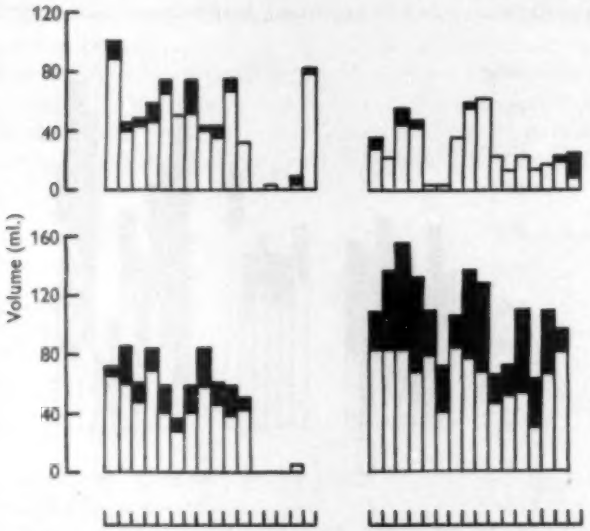



Fig. 8. Quantities of duodenal contents flowing in successive 5-min periods in four experiments on one sheep. The black areas show retrograde flow, the white areas the net amounts leaving the stomach.

TABLE 3. Comparison of flow rates between morning and afternoon experiments on goat 31

| Experiments between 9.0 a.m. and 2.0 p.m. | | Experiments between 2.0 and 5.0 p.m. | |
|---|---|---|---|
| Time of commencement | Net aboral flow (ml./hr) | Time of commencement | Net aboral flow (ml./hr) |
| 9.35 | 208 | 2.20 | 988 |
| 11.30 | 348 | 2.25 | 720 |
| 11.40 | 780 | 2.25 | 950 |
| 11.50 | 568 | 2.25 | 914 |
| 11.50 | 711 | 2.25 | 1100 |
| 1.0 | 270 | 2.45 | 1209 |
| 1.0 | 174 | 2.50 | 804 |
| 1·0 | 499 | 2.55 | 1146 |
| Mean | 444 | Mean | 879 |
| s.d. | 230 | s.d. | 215 |

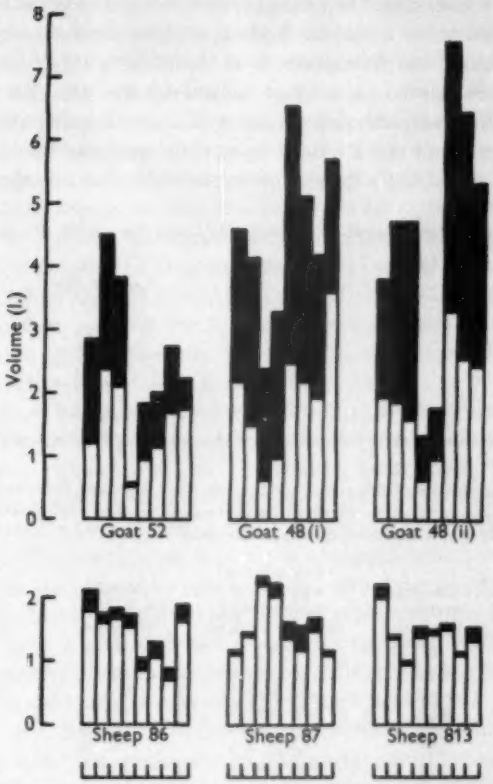



**Fig. 9. Histograms showing the pattern of flow over a composite 24 hr, in eight 3-hr periods, beginning with 10.30 p.m.–1.30 a.m. recorded on different days. Above are three results from two goats all showing a period of reduced flow in the early morning. Below are the results from three sheep which show no consistent pattern. Retrograde flow is black, net aboral flow white.**

**Table 4. The volume of duodenal flow in 24 hr obtained by adding eight 3-hr periods covering every hour of the day**

| Animal | Body wt. (kg) | Food intake: Hay (g) | Food intake: Concentrates (g) | Aboral flow (ml./24 hr) | Retrograde flow (ml./24 hr) | Net aboral flow (ml./24 hr) | Mean hourly flow (ml.) |
|---|---|---|---|---|---|---|---|
| Goat 52 | 33 | 581 | 267 | 21,024 | 8,661 | 12,363 | 515 |
| Goat 48 (i) | 33 | 900 | 400 | 37,506 | 21,439 | 16,067 | 669 |
| Goat 48 (ii) | 34 | 780 | 400 | 36,314 | 20,878 | 15,436 | 643 |
| Sheep 86 | 60 | 596 | — | 12,644 | 1,505 | 11,139 | 464 |
| Sheep 87 | 68 | 781 | — | 13,140 | 1,304 | 11,836 | 493 |
| Sheep 813 | 62 | 627 | — | 11,991 | 755 | 11,236 | 468 |

early morning which might be related to the unequal interval between feeds, although the sheep on a similar feeding routine show no regular pattern.

The large amount of retrograde flow shown by goat 48 in both experiments is in part due to an artifact caused by the use of a different preamplifier which had an unbalanced input. This produced spurious potentials due to movements of the animal but as they were randomly positive and negative they make little difference to the value for net aboral flow.

TABLE 5. Replicate recordings on sheep 929 over the period 10 a.m.–1.0 p.m.

| Aboral flow (ml.) | Retrograde flow (ml.) | Net aboral flow (ml.) |
|---|---|---|
| 1358 | 172 | 1188 |
| 1631 | 294 | 1337 |
| 1369 | 160 | 1209 |
| 1800 | 201 | 1599 |
| 2067 | 662 | 1405 |

Mean net aboral flow 1162 ml. ± S.D. 144, coefficient of variation 12·4 %.

TABLE 6. Total quantities of dry matter and crude fibre digested, calculated from food and faeces analysis, and proportion digested in the reticulo-rumen, calculated from duodenal flow measurement and analysis of duodenal contents

| Animal | Total dry matter digested (g) | Dry matter apparently disappearing in the stomachs (g) | Total crude fibre digested (g) | Crude fibre apparently digested in the stomach (g) |
|---|---|---|---|---|
| Goat 52 | 425 | 239 (56 %) | 100 | 101 (101 %) |
| Goat 48 (i) | 702 | 444 (63 %) | 154 | 146 (95 %) |
| Goat 48 (ii) | 679 | 369 (54 %) | 164 | 138 (83 %) |
| Sheep 86 | 376 | −80 (−21 %) | 96 | 48 (48 %) |
| Sheep 87 | 447 | 92 (21 %) | 98 | 77 (79 %) |
| Sheep 813 | 341 | 140 (41 %) | 155 | 129 (83 %) |

## DISCUSSION

The results of the calibration experiments indicate that the electromagnetic method of flow measurement is extremely accurate and that when the calibrations are performed on the animal, so that all possible error of measurement is included, the total error is negligible. Accepting that the measurements themselves are accurate, other objections can be raised as to the validity of the results. Transection and cannulation of the duodenum might affect the mechanisms which control the emptying of the stomach. In particular, the backward and forward movement of digesta in the duodenum might be a feature of this preparation, and may not occur in the intact animal. It is well known, however, that backward and forward movements have been observed radiologically in the duodenum of man, and there is no reason to doubt their occurrence in animals. The

return of food intake and faeces output to pre-operative levels after the animals had recovered from the surgical interference, and the maintenance of body weight, suggest either that the animals were not unduly affected or that they were rapidly adjusted to the condition. In addition, long periods occurred, particularly in the sheep, when the retrograde flow was almost negligible and the independent variation of retrograde flow might mean that it is part of the mechanism which controls the rate of stomach emptying. The to-and-fro movement may also be significant with regard to the point of entry of pancreatic secretion and bile, which is about a foot beyond the pylorus, in that these secretions would be carried forward and so would affect the pH in the first part of the duodenum. The results obtained from sheep are too few to justify the conclusion that as a species they differ from goats. The smaller amount of backward and forward movement could well be due to the difference in diet.

The methods of measurement used by Phillipson (1952) and by Hogan & Phillipson (1960) would prevent the occurrence of retrograde flow, and so might alter the values obtained. Expressing retrograde flow as a percentage of total aboral flow occurring over periods of at least an hour and ignoring the results obtained on goat 48 where artifacts occurred, a mean value of 40% with a range of 2–56%, is obtained for retrograde flow in goats, while for the sheep the mean is 5%, with a range of 0–17%.

The total quantity of material leaving the stomach in 24 hr will depend not only on the size of the animal and on its food and water intake, but also on the amount of digestion and absorption occurring in the reticulo-rumen and the volumes of salivary and gastric secretions, which in turn will be influenced by the composition of the food. An ideal method of measurement would be applicable to the animal without producing any disturbance of its normal behaviour, so that recordings could be made over several successive days without the animal being moved from its usual accommodation.

The method described here, by which the daily output is estimated by adding together the quantities recorded in 3-hr periods, assumes that (1) flow during a given 3-hr period does not vary significantly from day to day and (2) the volume of flow measured in the laboratory is representative of what would occur if the animal had not been disturbed. The first assumption was tested by performing replicate recordings of the flow over the same 3-hr period of successive days. The results are shown in Table 5. If we assume that all periods would have a similar coefficient of variation, and that each period is independent of the others, an estimate of the error from this source can be made for each experiment thus

$$\text{S.E. (24 hr)} = C\sqrt{(\Sigma_1^8 x^2)},$$

where $C$ = coefficient of variation, $x$ = volume flowing in 3-hr period. Calculated in this way the S.E. of the six 24-hr experiments lie between $\pm 4{\cdot}5$ and $\pm 4{\cdot}8\%$ of the totals.

Alternatively, if we assume that all 3-hr periods would have a similar standard deviation rather than $C$, the S.E. of the total would be

$$\sqrt{n_p}(\text{S.D.}/\sqrt{n_r}),$$

where $n_p$ = number of periods added together, $n_r$ = number of replicates. By this method, the S.E. would be $\pm 211$ ml., which is less than 2% of the 24-hr flow values.

The second assumption, that flow recorded in the laboratory is not different from what would occur if the animal were undisturbed, cannot be tested directly, but an assessment of the total error can be made by comparing the quantities of dry matter and crude fibre apparently digested in the stomachs with the results of other workers obtained by different methods. The values of Table 6 have been calculated from results of chemical analyses of food, duodenal contents and faeces which are to be presented in another paper.

Hogan & Phillipson (1960), using a similar preparation, measured duodenal flow directly in sheep by collecting material from the lower cannula, measuring its volume and reintroducing it into the upper cannula at short intervals. They found a mean flow rate of 360 ml./hr through the duodenum. On the basis of this measurement they report that 70% of the digested dry matter was digested in the stomach. The sheep were fed on a diet similar to that of the goats in the present experiments, which show an apparent disappearance of only 54–63% of the dry matter in the stomachs. This lower value could be accounted for by the 24-hr flow estimates being too high, but the proportions of the crude fibre digested which disappear between mouth and pylorus suggest that they are, if anything, slightly low.

Gray (1947), using a lignin-ratio technique, reported that 70% of the cellulose digested in sheep was digested in the rumen, and Hale, Duncan & Huffman (1940) by a similar method found a corresponding value of 85% in cows. It is surprising that Hogan & Phillipson should obtain a relatively low value for duodenal flow, as their method of measurement would prevent retrograde flow and they removed 10% of each sample before returning it to the duodenum, which would tend to increase the rate of stomach emptying.

The sheep in the present experiments were fed on hay only, so that in the absence of readily fermentable concentrate it is to be expected that less of the digestible dry matter would disappear in the reticulo-rumen. The result for sheep 86 appears to be a considerable over-estimate of duodenal flow, as more dry matter appears at the duodenum than was eaten. This in

itself is not impossible, since the dry matter of duodenal contents contains 14–25 % ash (Masson & Phillipson, 1952) compared with 5–7 % in that of hay, and other constituents of saliva and gastric juice may be present. By comparison with the rumenal digestion of crude fibre in the other two sheep it would appear to be an over-estimate of about 27 %. This sheep had a particularly nervous temperament, and it may well be that duodenal flow was greater in the laboratory than when the animal was undisturbed.

## SUMMARY

1. An electromagnetic method of recording the flow of digesta through the duodenum of small ruminants is described.

2. The flow pattern shows peristaltic-antiperistaltic movement; in goats about 40 % of the material leaving the stomach is returned to it and in sheep about 5 %.

3. In periods of up to 1½ hr of recording during the day the mean net aboral duodenal flow ranged from 400 to 1300 ml./hr for different goats.

4. Estimates of the total flow through the duodenum per day were made in six separate experiments carried out on two goats and three sheep. Goats on a diet of hay and concentrates had a duodenal flow rate of 12–15 l./day, and sheep on a diet of hay 11 l./day.

5. The errors involved in these estimations are discussed.

I wish to thank Drs T. G. Richards and T. D. Williams for helpful advice on apparatus, Messrs J. McCarthy and J. Stowell for technical assistance, and Mrs A. P. Ridges who assisted with part of the 24 hr recording. Special thanks are due to Professor E. G. White for his continued interest in these experiments, and for the provision of accommodation in the Department of Veterinary Preventive Medicine. The work was supported by a grant from the Agricultural Research Council.

## REFERENCES

Gray, F. V. (1947). The digestion of cellulose by sheep. *J. exp. Biol.* **24**, 15–19.

Hale, E. B., Duncan, G. W. & Huffmann, C. F. (1940). Rumen digestion in the bovine with some observations on the digestibility of Alfalfa Hay. *J. Dairy Sci.* **23**, 953–967.

Hogan, J. P. & Phillipson, A. T. (1960). The rate of flow of digesta and their removal along the digestive tract of the sheep. *Brit. J. Nutr.* **14**, 147–155.

Katz, I. N. & Kolin, A. (1938). The flow of blood in the carotid artery of the dog under various circumstances as determined with the electromagnetic flowmeter. *Amer. J. Physiol.* **122**, 788–804.

Masson, M. J. & Phillipson, A. T. (1952). The composition of the digesta leaving the abomasum of sheep. *J. Physiol.* **116**, 98–111.

Phillipson, A. T. (1952). The passage of digesta from the abomasum of sheep. *J. Physiol.* **116**, 84–97.

Richards, T. G. & Williams, T. D. (1953). Velocity changes in the carotid and femoral arteries of dogs during the cardiac cycle. *J. Physiol.* **120**, 257–266.

Wetterer, E. (1937). Eine neue Methode zur Registrierung der Blutströmungsgeschwindigkeit am uneröffneten Gefäss. *Z. Biol.* **98**, 26–36.

*J. Physiol.* (1961), **155**, *pp.* 148–160
*With 7 text-figures*
*Printed in Great Britain*

# ANALYSIS OF THE ANTIDROMIC CORTICAL RESPONSE FOLLOWING STIMULATION AT THE MEDULLARY PYRAMIDS

BY S. J. JABBUR AND A. L. TOWE

*From the Department of Physiology and Biophysics, University of Washington School of Medicine, Seattle, Washington, U.S.A.*

(*Received* 22 *August* 1960)

The electrical response recorded from the surface of the pericruciate cortex following stimulation of the medullary pyramids was first investigated by Woolsey & Chang (1948), and was studied in further detail by Chang (1955*a*, *b*), Landau (1956) and others. This response has been used to map the origin of the pyramidal tract (Woolsey & Chang, 1948; Lance & Manning, 1954; Porter, 1955) and as an index of activity in various parts of the cortical pyramidal neurones (Woolsey & Chang, 1948; Chang, 1952, 1955*a*, *b*; Landau, 1956; Purpura & Grundfest, 1956). Largely because antidromic invasion is thought to be a sequential process, the axon, cell body and proximal portion of the apical dendrite have been implicated in the initial positive deflexion and the superficial portion of the apical dendrite has been held responsible for the subsequent negative wave. The complex array of basal dendrites has been ignored. A late, large potential, usually surface-negative in the motor cortex, has been said to result from recurrent collateral activation of the superficial portion of the apical dendrites. Landau (1956) has taken issue with this interpretation, believing that the latter is an orthodromic response in the cortex produced by current spread to the adjacent medial lemniscus. All these studies have involved electrical stimulation of the medullary pyramids *in situ*. In order to analyse the cortical response with a view to defining which components result from antidromic activity in the pyramidal tract and which components depend upon spread of current to adjacent structures, strands of medullary pyramid were stimulated when they were in contact with, and when they were isolated from, the brain stem (Towe & Jabbur, 1959).

## METHODS

Of the forty cats used in this study, thirty-four were anaesthetized with α-chloralose (35–40 mg/kg, I.P.) and immobilized with decamethonium bromide (1 mg/hr, I.V.), and six were anaesthetized with pentobarbital sodium (35 mg/kg, I.P.) and immobilized, when necessary, with D-tubocurarine chloride (1 mg/hr, I.V.). All animals were maintained on

artificial respiration; bilateral pneumothorax was produced in thirty cats. The bulbar pyramids were exposed by a ventral approach, and the anterior portions of both cerebral hemispheres were exposed in a routine manner. During the experiment a portion of one medullary pyramid was dissected out as a strand 0·5–0·7 mm thick, 1–2 mm wide and 7–10 mm long, and severed transversely at its caudal end. This strand could be lifted free from the underlying tissue and stimulated in mineral oil or in air to excite the cortical pyramidal cells antidromically. The extent of the strand dissection was estimated from Luxol-fast-blue and Weil-stained sections of the formalin-perfused brain stem. Extreme caution was required in dissecting the pyramidal strand, because fibres of the medial lemniscus, passing ventrally to the inferior olive, are juxtaposed to pyramidal fibres for a

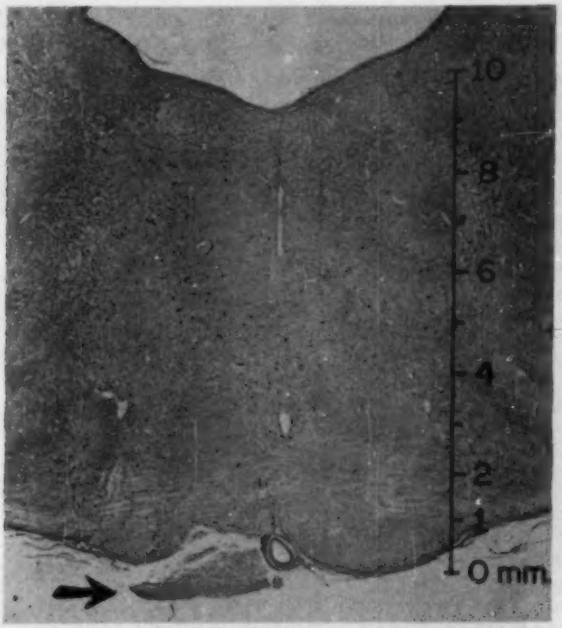



Fig. 1. Luxol-fast-blue stained section through posterior trapezoidal region of brain stem, showing extent of isolated strand dissection (arrow) in typical preparation. The millimetre scale on the left has been adjusted for a 37 % shrinkage in this paraffin-base preparation.

centimetre of their course in the bulb (Glees, Liddell & Phillips, 1951). The histological preparations did not allow differentiation of these two fibre tracts; the geometric relations of the dissection constituted the only anatomical clue to the success or failure of the dissection (Fig. 1).

Bipolar needle electrodes were inserted into the central foot pads of the forepaws, and 0·1 msec square-current pulses were induced by means of radio-frequency isolation units. Both bipolar silver-wire and concentric steel electrodes were employed for stimulation of the bulbar pyramids. Hooked silver-wire electrodes were used to elevate the dissected strand

free from the underlying tissue (the animal was maintained supine). With a micrometer drive system the strand could be raised and lowered during an experiment and could thus be stimulated while isolated from or in contact with the brain stem. Square-current pulses, usually 0·01 msec in duration, but occasionally longer, were delivered to the pyramidal strand and the brain stem.

A silver-ball surface-monitoring electrode was placed on arm area I of the sensorimotor cortex to record the evoked potentials. Conventional recording methods were employed; the over-all low-frequency time constant of the recording system was kept around 140 msec.

## RESULTS

A single shock to the surface of the intact medullary pyramids elicited in the ipsilateral sensorimotor cortex a complex potential consisting of three or four distinct components. The first of these components, labelled *a* in Fig. 2, began 0·4–0·6 msec after the bulbar shock and lasted about 1 msec.

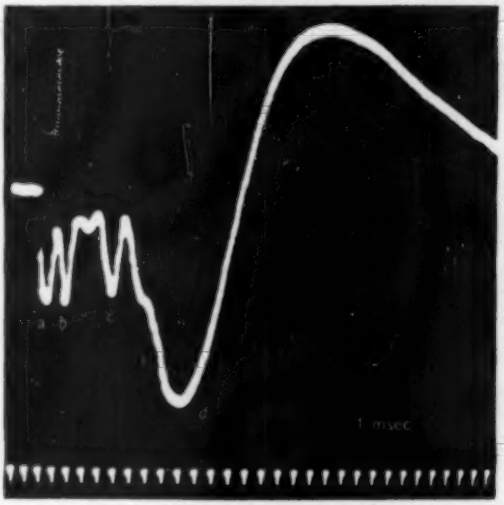



Fig. 2. Response at surface of postcruciate cortex, about 1 mm posterior to the lateral tip of the cruciate sulcus, following stimulation of the surface of the medullary pyramids through bipolar electrodes. Pulse duration, $p$ = 0·05 msec; pulse intensity, $i$ = 10 potentiometric units (one potentiometric unit equals about 1·2 V at the output side of the isolation transformer). In this and all subsequent figures, positivity of the recording lead with respect to the indifferent lead is down. Peak-to-peak amplitude of the *d* wave is 0·8 mV.

It faithfully followed repetitive shocks in excess of 400/sec, was the last component to fail during asphyxiation and, of all the components, had the lowest threshold. The *b* component had a latency of about 1·5 msec and, like *a*, lasted 1 msec. Unlike *a*, however, *b* failed to follow repetitive

shocks at frequencies exceeding 80–100/sec. The sensitivity of component *b* to asphyxia was between that of *a* and *c*. Component *c* had a much higher threshold than *a*, a variable configuration from one experiment to the next, and failed to follow repetitive shocks at frequencies above 10–20/sec. The last component, labelled *d*, had properties similar to those of the primary evoked response that followed stimulation of the contralateral cutaneous surface. In fact, at some loci on the pericruciate cortex, the primary evoked response and the *d* component were almost identical. The time course of recovery of excitability of *d* following a single shock to the ventral brain stem was identical with that of the primary response following a single cutaneous shock. Furthermore, *d* responses and primary

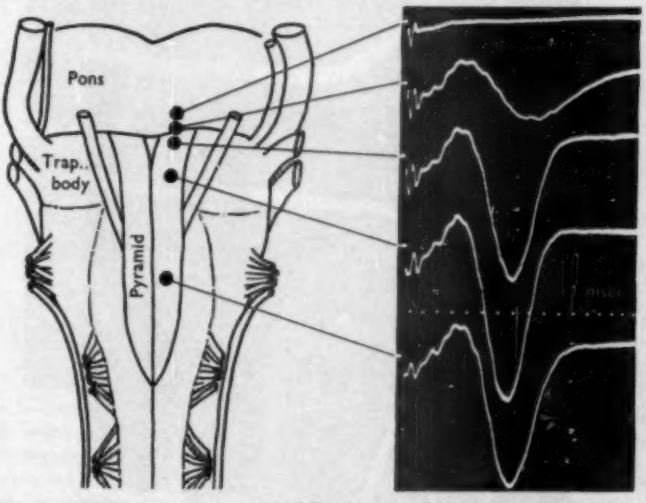



Fig. 3. Postcruciate cortical responses following stimulation of the unoperated brain stem at the various positions indicated on the drawing of the ventral brain stem ($p$ = 0·05, $i$ = 10). In uppermost trace, $a$ and $\beta$ are present, but $b$, $c$ and $d$ are not produced at this intensity. Increasing the stimulus intensity at the most rostral position produces a wave identical to the next lower trace.

responses interacted in precisely the same way, as did two primary responses or two *d* responses. It was concluded that the *d* component resulted from current spread to, and activation of, the medial lemniscus.

If the *d* component results from orthodromic lemniscal input, it is tempting to regard the *a*, *b*, and *c* components as the consequences of antidromic activity in pyramidal tract axons. If this interpretation is correct, stimulation of the pyramidal tract alone should yield all but the *d*, or lemniscal, wave. Stimulation of the pyramidal tract alone should be

possible by weakening the shock strength or by stimulating in a region (e.g. the pons) where the medial leminiscus and the pyramidal tract diverge. However, as is shown in Fig. 3, when the stimulus position was advanced to the pons, not only the *d* wave, but also the *b* and *c* waves diminished and disappeared. At the most rostral stimulus site strengthening the shock recaptured the later components, while weakening the shock further reduced the *a* response. Note in the uppermost trace of Fig. 3 and trace *A* of Fig. 4 that a second, prolonged positive wave is present. The fast deflexion of the *b* wave is superimposed upon this wave; the prolonged

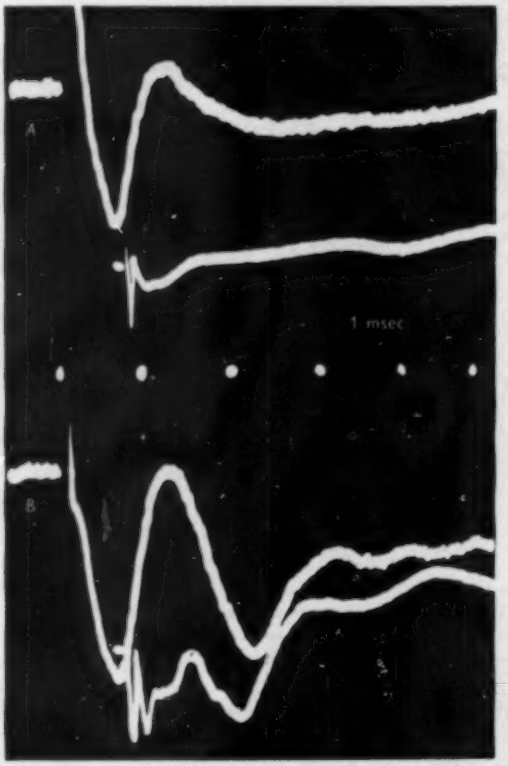



Fig. 4. *A*: Pyramidal antidromic response obtained by very weak stimulation ($p$ = 0·04 msec, $i$ = 10) over the rostral trapezoid region of the unoperated brain stem. The *a* wave is 0·3 mV, *β* is 0·1 mV. *B*: Same stimulus strength applied over the middle of the pyramids (mid-olivary level) produces late components. The *a* wave is almost 0·5 mV, *b* about 0·25 mV. Time applies to fast sweeps; slow sweep speed is 1/10 of fast sweep speed. First 3 msec of both slow sweeps retouched.

portion may be a separate component. Hereafter we shall restrict the term *b* wave to the fast deflexion and use the term $\beta$ to refer to the small, prolonged positive wave that follows *a*. Comparison of the slow and the fast sweeps, *A* and *B* of Fig. 4, reveals the *b* and $\beta$ components, and suggests that *c* was surface-negative in that particular preparation.

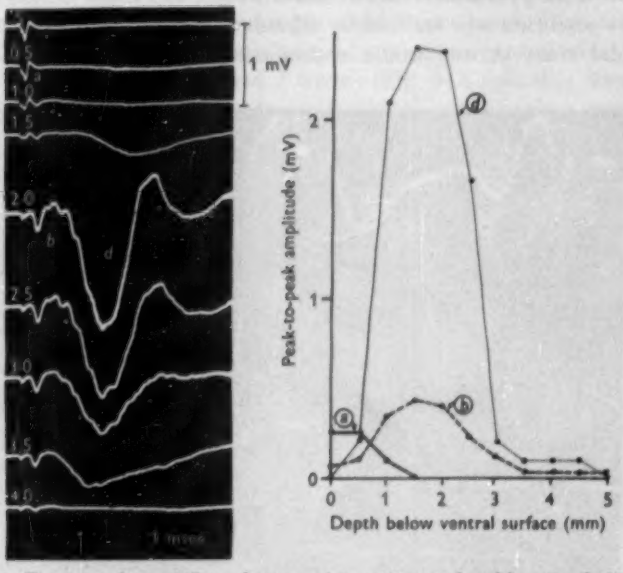



Fig. 5. Responses from surface of posteruciate cortex produced by stimulation at various depths in the brain stem with a coaxial electrode. Numbers indicate depth beyond ventral surface of pyramids according to manipulator vernier (see Fig. 1). The traces below 2·0 mm show the shock artifact but no *a* response. Series taken during *penetration* from surface; $p$ = 0·025 msec, $i$ = 10. Graph on right side plots amplitudes of *a*, *b*, and *d* as a function of position of bipolar electrode during *withdrawal* to surface; $p$ = 0·01 msec, $i$ = 10. The amplitudes of *a* and *b* were measured from fast, high-gain sweeps (not illustrated), whereas *d* was measured from simultaneous slow, low-gain sweeps such as those shown in the traces on the left. The amplitude of *b* includes $\beta$; however, $\beta$ did not exceed 50 $\mu$V in this preparation (see point on *b* curve at zero depth), and hence does not severely distort the graph.

The *b* and *c* components, like the *d* wave, appeared to depend upon spread of the current to structures beyond the pyramidal tract. Local stimulation at various depths in the brain stem aided in identifying the fibres responsible for the *b* wave (Fig. 5). During penetration and withdrawal of either bipolar or concentric stimulating electrodes, the various components of the cortical response altered in amplitude in a characteristic way. The fibres producing the *b* component are located in the region of the

medial lemniscus, and extend a little more dorsally. The *c* wave was not measured.

The above analysis implies that if the pyramidal tract is stimulated in isolation and without significant spread of current to adjacent structures, then only *a* and $\beta$ will appear. Further, stimulating the base of the bulb exclusive of the pyramidal tract should elicit *b*, *c*, and *d*, but neither *a* nor $\beta$. The first condition was realized by stimulation of the dissected strand of pyramidal tract as one would a desheathed peripheral nerve bundle.

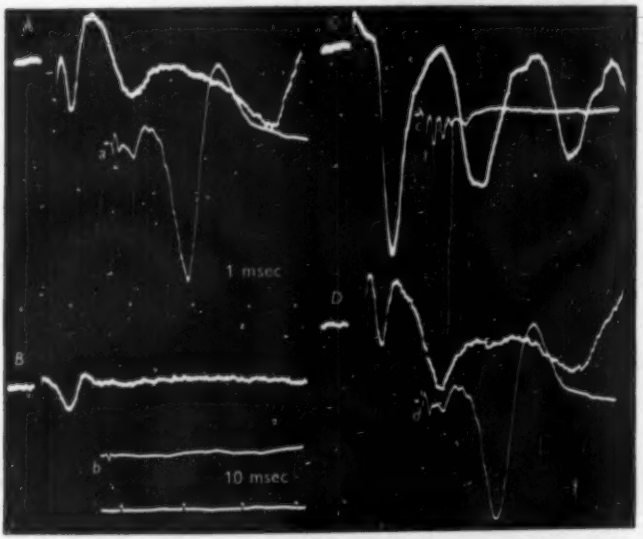



Fig. 6. Recording 2 mm posterior and medial to lateral tip of cruciate sulcus. *A*: Stimulation of pyramidal strand and subjacent brain stem; $p = 0{\cdot}04$ msec, $i = 10$. *B*: Stimulation of isolated pyramidal strand; *a* wave is 75 $\mu$V in amplitude, $p = 0{\cdot}10$ msec, $i = 10$. *C*: *D* and *I* waves recorded from distal electrode on isolated strand following direct cortical stimulation. *D*: Shape of cortical potential before operation; $p = 0{\cdot}04$ msec, $i = 10$. *a*, *b*, *c* and *d* show the slow sweeps recorded simultaneously.

Traces *A* and *a* in Fig. 6 show the entire complex produced by bipolar stimulation of such a strand lowered into place in the brain stem; all four components are evident. When the strand was raised above the underlying tissue (Fig. 6*B* and *b*), only the *a* wave and a slight $\beta$ wave could be elicited. The *a* wave thus produced was always smaller than that produced in the undissected preparation or when the strand lay on the brain stem, probably because the strand included only a part of the pyramidal tract.

Maximal stimulation of the pyramidal strand (10 msec square pulse of 12 V amplitude) failed to recruit the *b*, *c* and *d* components. The isolated *a* wave followed stimulus repetition rates in excess of 400/sec with no attenuation, but maintaining its simple configuration. No subsequent components appeared as the stimulus rate was varied from 0·2 to 500/sec. Each isolated strand remained viable for 1–4 hr and then deteriorated rapidly, so that the *a* wave no longer could be produced. The viability of a freshly prepared strand was indicated by its capacity to conduct corticofugal discharges such as *D* and *I* waves (Fig. 6*C*) resulting from direct cortical stimulation (Patton & Amassian, 1954) and the reflex corticofugal discharge following cutaneous stimulation (Patton & Amassian, 1960). The fact that lowering the strand into place and stimulating the brain stem resulted in an *a* wave of the same amplitude as the unoperated response (compare traces *A* and *a* with *D* and *d* of Fig. 6) further attests the viability of the strand.

In different preparations the amplitude of the *a* wave produced by stimulation of the isolated pyramidal strand ranged from 15 to 90% (usually 50–70%) of the amplitude of the *a* wave elicited by stimulating the ventral pyramids of the unoperated preparation. This variation was principally due to differences in the sizes of the dissected strands. The $\beta$ waves were small, being almost undetectable in half of the preparations but clearly present in the others.

In several preparations the strand was elevated out of the way, the rest of the pyramidal tract was removed, and the remaining brain stem was then stimulated with bipolar electrodes. Such stimulation produced the *b*, *c*, and *d* waves, without the *a* wave. In Fig. 7*A* the $\beta$ wave can be detected, but it is absent from Fig. 7B. This can be seen best in the slow sweeps, comparing the voltage at the end of *b* in the two sweeps. The *c* wave in this preparation was surface-positive.

In summary, antidromic pyramidal activity produces the *a* and $\beta$ waves. Orthodromic medial-lemniscus activity produces the *d* wave and activity in certain fibres intermingled with the medial lemniscus produces the *b* wave. The elusive *c* wave does not result from pyramidal tract activity; its origin remains obscure.

## DISCUSSION

The results now presented differ from those of previous similar studies in that the 'pyramidal antidromic response' has been reduced to a simple, two-component, positive response. This two-component portion of the more complex response produced in the pericruciate cortex by bulbar stimulation can be detected in published records (Woolsey & Chang, 1948; Lance & Manning, 1954; Porter, 1955; Chang, 1955*a*, *b*; Landau, 1956) of other investigators. However, components *b* and *c* have been erroneously

included as components of the pyramidal antidromic response. Comparison of the cortical responses to pyramidal stimulation in the different species studied by Woolsey & Chang (1948) and by Porter (1955) reveals the *a* wave to be the only clear common feature. We have also stimulated an isolated pyramidal strand in a squirrel monkey; a cortical response identical with that in the cat was obtained. It thus appears that the simple, two-component configuration is a common feature of antidromic cortical responses of mammals.

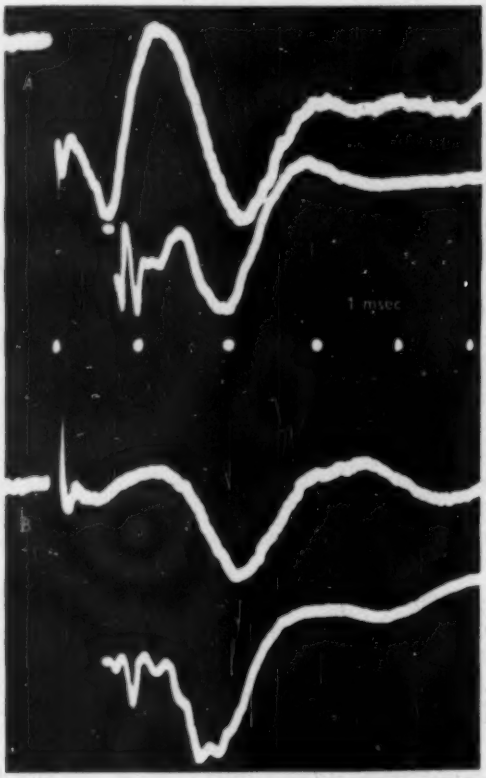



Fig. 7. Records from posteruciate cortex following bipolar stimulation of brain stem with and without the pyramidal tract; $p$ = 0·04 msec, $i$ = 10. *A*: simultaneous stimulation of pyramidal strand and subjacent brain stem. *B*: stimulation of brain stem with pyramidal strand deflected forward, and hence not stimulated. Both *a* and $\beta$ are absent in this trace, but *b* remains. Time applies to fast sweep; slow sweep speed 1/10 of fast sweep speed. The first 3 msec of both slow sweeps have been retouched.

The present experiments do not indicate whether or not apical dendrites are depolarized during antidromic invasion. If apical dendrites are actively invaded during antidromic activation, however, they make no significant contribution to cortical surface negativity; the $a$ and $\beta$ components are preponderantly surface-positive. This conclusion is contrary to the assertion (Chang, 1952, 1955*b*) that apical dendrites produce a large, surface-negative potential during antidromic invasion. There is also reason to doubt that surface-negative components of orthodromic cortical potentials result from superficial dendritic depolarization of pyramidal tract neurones. Von Euler & Ricci (1958) found that the positive and the negative components of the evoked primary response in the auditory cortex are independent of each other. Patton & Towe (1957; Towe & Patton, 1957; A. L. Towe, unpublished observations) have studied both the rate of change of potential with depth and the time–depth distribution of unit activity in the same patch of somatosensory cortex. They found a striking agreement between unitary activity and potential distribution at all levels within the cortex. The proposal that surface-negativity depends upon depolarization of apical dendrites is inconsistent with these observations. It is interesting to note, in this connexion, that the hippocampal surface response to direct hippocampal stimulation (Kandel, Spencer & Brinley, 1960) is similar to the direct cortical response obtained by stimulation of the surface of the cerebral cortex (Chang, 1952) even though the soma-dendritic organization is inverted.

The simplicity of the cortical response following stimulation of the isolated pyramidal strand argues strongly against recurrent collateral activity measurable at the cortical surface. Once the stimulus intensity is just maximal for $a$, the response in the isolated strand is not altered either by a further increase of stimulus intensity or by repetition of the stimulus at frequencies between 0·2/sec and 500/sec ($\beta$ is obscured at very high frequencies by superposition of $a$). No late responses ascribable to recurrent collaterals could be discerned, even in unparalysed preparations anaesthetized with pentobarbital sodium (see Purpura & Grundfest, 1956).

The pyramidal tract of the cat contains two fibre populations (Brookhart & Morris, 1948; Bishop, Jeremy & Lance, 1953; Lance, 1954), having mean conduction velocities of approximately 60 m/sec and 20 m/sec respectively. Both groups show a wide distribution of conduction velocities, the combined range being from about 8 m/sec to over 100 m/sec. Computed mean conduction rates of $a$ and $\beta$ in the present experiments (assuming a conduction distance of 5 cm and measuring latencies to peak voltages) were 55 m/sec and 17–25 m/sec respectively. The $\beta$ component has a long duration and encloses a large time–voltage area compared with $a$. This makes the time to peak amplitude of $\beta$ difficult to measure. Measure-

ment is most difficult in responses to isolated pyramidal strand stimulation, for $\beta$ is then very small. The small size of $\beta$ in such responses may result from greater injury to the smaller fibres during and after dissection.

The 1 msec duration of *a* allows a fibre spectrum ranging over 12 $\mu$. On the other hand, the longer latency of the 1 msec duration *b* wave requires that the fibres mediating it should range less than 3 $\mu$, from smallest to largest. This calculation is based on the assumption that *b* is also of antidromic origin. It has been shown that the fibres producing *b* are distributed throughout the medial lemniscus. The capacity of *b* to follow high-frequency repetitive stimuli, its resistance to asphyxia (nearly equal to that of *a*), and its predominantly surface-positive configuration all suggest that *b* results from antidromic conduction along fibres lying outside the medullary pyramids and projecting at least as far caudally as the level of the inferior olivary nucleus. The requisite fibres are 3–6 $\mu$ in diameter and exist in abundance, for *b* has a greater amplitude than *a*. A possible alternative explanation for the relatively large size of *b* is that the *b* elements lie closer to the cortical surface than the *a* elements. Considerable anatomical study will be required to determine whether the *b* system is an 'aberrant' pyramidal tract (Winkler, 1927), a corticobulbar tract, or some long tract hitherto undefined.

The records presented in this paper were obtained almost exclusively from a 4 $mm^2$ patch of cortex centred about 1·8 mm posterior to the lateral tip of the cruciate sulcus; this is in arm area I of the sensorimotor cortex. Similar responses have been recorded throughout the pericruciate cortex, the most significant variation being in the configuration of *d*, the late lemniscal wave. The density of cells whose axons project into the medullary pyramids is nearly as great in the postcruciate region, from which the illustrations were drawn, as in the precruciate cortex (Patton & Amassian, 1960). It is no surprise, therefore, to find that postcruciate *a* responses are as large as (or sometimes larger than) those recorded from sites rostral to the cruciate sulcus.

The origin of *c* is obscure. It has some attributes of an antidromic response. Although *c* disappears at low rates of stimulus repetition, it is almost as resistant to asphyxia as is *b*. In some experiments *c* is lacking. When present, it takes any of several configurations. Its duration is sometimes surprisingly short for such a late potential. Often, the time rate of change of voltage is as great for *c* as for *a*. Such extreme synchrony in a synaptic system would be remarkable.

SUMMARY

1. The electrical response recorded at the surface of the pericruciate cortex following stimulation of the ventral brain stem at the level of the medullary pyramids has been analysed in the cat. Various methods have been employed to differentiate the different components of the cortical response.

2. The observations showed that three components were produced because current spread beyond the pyramidal tract and excited other fibre systems.

3. The pyramidal tract was then dissected free from adjacent tissue, except at its rostral end, and stimulated with minimal current spread outside the strand. The resultant response of the cortex consisted of a surface-positive 'spike' followed by a longer-duration, smaller, positive wave. This is the pyramidal antidromic response; *nothing more* depends upon antidromic pyramidal tract activity.

4. The additional potential changes that are present when the intact pyramidal tract is stimulated result from excitation of elements outside the medullary pyramids; these additional components were produced without the antidromic pyramidal response by stimulation of the brain stem after complete removal of the bulbar pyramidal tract.

This investigation was supported by a grant (B396) from the National Institute of Neurological Diseases and Blindness, Department of Health, Education and Welfare and a grant from the State of Washington Research Fund for Biology and Medicine.

REFERENCES

Bishop, P. O., Jeremy, D. & Lance, J. W. (1953). Properties of pyramidal tract. *J. Neurophysiol.* **16**, 537–550.

Brookhart, J. M. & Morris, R. E. (1948). Antidromic potential recordings from the bulbar pyramid of the cat. *J. Neurophysiol.* **11**, 387–398.

Chang, H.-T. (1952). Cortical neurons with particular reference to the apical dendrites. *Cold Spr. Harb. Symp. quant. Biol.* **17**, 189–202.

Chang, H.-T. (1955*a*). Cortical response to stimulation of medullary pyramid in rabbit. *J. Neurophysiol.* **18**, 332–352.

Chang, H.-T. (1955*b*). Activation of internuncial neurons through collaterals of pyramidal fibres at cortical level. *J. Neurophysiol.* **18**, 452–471.

Glees, P., Liddell, E. G. T. & Phillips, C. G. (1951). Der Verlauf der medialen Schleife im Hirnstamm der Katze. *Z. Zellforsch.* **35**, 487–494.

Kandel, E. R., Spencer, W. A. & Brinley, F. J. (1960). Transient and long-lasting electrical responses to direct hippocampal stimulation. *Amer. J. Physiol.* **198**, 687–692.

Lance, J. W. (1954). Pyramidal tract in spinal cord of cat. *J. Neurophysiol.* **17**, 253–270.

Lance, J. W. & Manning, R. L. (1954). Origin of the pyramidal tract in the cat. *J. Physiol.* **124**, 385–399.

Landau, W. M. (1956). An analysis of the cortical response to antidromic pyramidal tract stimulation in the cat. *Electroenceph. clin. Neurophysiol.* **8**, 445–456.

Patton, H. D. & Amassian, V. E. (1954). Single- and multiple-unit analysis of cortical stage of pyramidal tract activation. *J. Neurophysiol.* **17**, 345–363.

PATTON, H. D. & AMASSIAN, V. E. (1960). The pyramidal tract: its excitation and functions. In *Handbook of Physiology, Section* 1, *Neurophysiology*, **2**, ch. 34, ed. FIELD, J., MAGOUN, H. W. & HALL, V. E. Washington, D.C.: American Physiological Society.

PATTON, H. D. & TOWE, A. L. (1957). Contributions of cellular elements to primary response of somatosensory cortex. *Fed. Proc.* **16**, 99.

PORTER, R. (1955). Antidromic conduction of volleys in pyramidal tract. *J. Neurophysiol.* **18**, 138–150.

PURPURA, D. P. & GRUNDFEST, H. (1956). Nature of dendritic potentials and synaptic mechanisms in cerebral cortex of cat. *J. Neurophysiol.* **19**, 573–595.

TOWE, A. L. & JABBUR, S. J. (1959). *Science*, **129**, 1676–1678.

TOWE, A. L. & PATTON, H. D. (1957). Unitary responses of 'Betz' cells to antidromic and orthodromic excitation. *Fed. Proc.* **16**, 129.

VON EULER, C. & RICCI, G. F. (1958). Cortical evoked responses in auditory area and significance of apical dendrites. *J. Neurophysiol.* **21**, 231–246.

WINKLER, C. (1927). *Manuel de Neurologie*, vol. 1, part 3. Haarlem: De Erven F. Bohn.

WOOLSEY, C. N. & CHANG, H.-T. (1948). Activation of the cerebral cortex by antidromic volleys in the pyramidal tract. *Res. Publ. Ass. nerv. ment. Dis.* **27**, 146–161.

*J. Physiol.* (1961), **155**, *pp.* 161–174
*With 7 text-figures*
*Printed in Great Britain*



# THE ACTION OF POSTERIOR PITUITARY HORMONES AND OESTROGENS ON THE VASCULAR SYSTEM OF THE RAT

BY SYBIL LLOYD AND MARY PICKFORD
*From the Department of Physiology, University of Edinburgh*

(*Received* 29 *August* 1960)

In two recent papers (Lloyd, 1959*a*, *b*) it was shown that the vascular responses of the rat to oxytocin and vasopressin varied with the concentration of ovarian hormones in the body. For example, oxytocin was dilator in the dioestrous rat, though without effect on the blood pressure, while in the oestrous animal, during late pregnancy, or after ovarian hormone administration, oxytocin was pressor and constrictor. It was not known how far this reversal of effect was due to an altered state of the peripheral vasculature, or how far central mechanisms were involved. Since peripheral vasodilatation is one of the consequences of the administration of oestrogen, it was of interest to test the effect of other dilator substances on the response to oxytocin and vasopressin in the rat, and for this purpose infusions of histamine, acetylcholine, isoprenaline and 5-hydroxytryptamine (5-HT) were used. In addition, procedures such as pithing, decerebration, and the administration of autonomic blocking agents were used to determine whether reduction of vasomotor tone would affect the responses to posterior pituitary hormones, and to discover if any part was played by the central and peripheral nervous systems.

## METHODS

All experiments were made on rats of approximately 200 g body weight. Anaesthesia, injection of drugs, and recording of blood pressure were effected by the methods previously described (Lloyd, 1959*a*, *b*). The stage of the reproductive cycle was checked in all females by vaginal smears stained with Leishmann stain. All drugs were dissolved in NaCl solution 0·9 g/100 ml. Single intravenous injections were made up to a volume of 0·3 ml.; intravenous infusions were made into a cannulated femoral vein, at a rate of 0·05 ml./min. Autonomic blocking agents were either given intravenously during the experiments, or subcutaneously 2–3 hr before observations were begun. In experiments in which rats were pre-treated with an oestrogen, stilboestrol dipropionate was used, given in a dose of 3·5 $\mu$g/100 g 24 hr before observations were made.

The oxytocin used was the synthetic brand Syntocinon (Sandoz). Previous experience showed that the actions of Syntocinon were identical with those of purified oxytocic extracts of the posterior pituitary (Brooks & Pickford, 1958; Nixon & Smyth, 1957). The vasopressin was either Parke Davis's Pitressin or du Vigneaud's highly purified arginine vasopressin. The bretylium tosylate (Darenthin) was supplied by the Wellcome Research Laboratories,

and the 5-hydroxytryptamine (as Serotonin creatinine sulphate) by Abbott Laboratories. The reserpine was the Ciba product Serpasil, and the ergot preparation Dihydroergotamine (Sandoz). Histamine was used in the form of the acid phosphate (British Drug House). The dibenamine solution was prepared by the method of Dekanski (1952).

## RESULTS

### *The effect of infusion of dilator substances*

The vasodilator substances used were acetylcholine, isoprenaline, histamine and 5-hydroxytryptamine. The doses and results are summarized in Table 1. All dilator drugs were administered by slow intravenous infusion lasting approximately 30 min, and the responses to oxytocin and vasopressin were tested before, during and after the infusions. The general blood pressure was lowered by as much as 20 mm Hg by infusion at the higher concentrations, though with the smaller doses any depressor action was temporary. In eleven experiments on dioestrous females up to

TABLE 1. The effect of intravenous infusions of vasodilators on the response to oxytocin

| Substance infused (μg/min) | Total no. of rats | Effect of oxytocin on blood pressure | |
|---|---|---|---|
| | | Pressor before, during and after infusions | No effect before, during or after infusions |
| Acetylcholine 0·05–1·0 | 6 | 2 (*F*, oestrous) | 4 (3*F*, dioestrous, 1*M*) |
| Isoprenaline 0·02–1·0 | 7 | 2 (*F*, oestrous) | 5 (2*F*, dioestrous, 3*M*) |
| 5-HT 0·5–1·0 | 5 | 1 (*F*, oestrous) | 4 (4*F*, dioestrous) |
| Histamine 2·5–5·0 | 4 | 1 (*F*, oestrous) | 3 (2*F*, dioestrous, 1*M*) |

*F* = female; *M* = male.

100 m-u. oxytocin had, as expected, no effect on the blood pressure before infusion of a dilator substance, and it was found that no pressor response to oxytocin appeared either during or for 2 hr after infusion of any of the dilator agents (Fig. 1*A*). In five experiments on normal males similar results were obtained; that is, no pressor response to oxytocin was observed before, during or after infusions of any of the vasodilator substances. The effect of the infusions on the response to vasopressin was also tested in most of these animals, and in no instance was the magnitude of the pressor response altered.

In six oestrous rats, in which a pressor response to oxytocin was present before infusion, the size and duration of this effect were unaltered by any of the dilator drugs (Fig. 1*B*). For example, 50 m-u. oxytocin raised the blood pressure by 10 mm Hg before, during and after the infusion of isoprenaline. Similarly, though the pressor effect of vasopressin was greater in this group of animals than in the dioestrous females or males, the size

of the response was unchanged by the infusions. Thus, if 0·2–0·3 m-u. vasopressin increased the blood pressure by 10 mm Hg before infusion, it raised it to the same extent during and after the infusion.

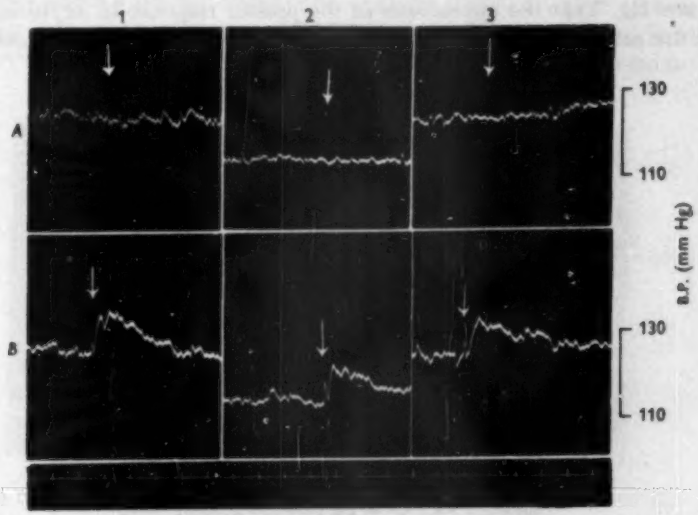



Fig. 1. The effect of 50 m-u. oxytocin on the blood pressure of (*A*) the dioestrous and (*B*) the oestrous rat, (1) before, (2) during, and (3) after the intravenous infusion of isoprenaline, 0·05 $\mu$g/min for 30 min. Time marker, 30 sec.

*The effect of surgical interference with the nervous system*

*Pithing.* Twenty-three rats were pithed after the normal responses to oxytocin and vasopressin had been established. The animals were maintained on artificial respiration. The results are summarized in Table 2. In eight dioestrous females and four males oxytocin initially had no effect on the blood pressure, but after pithing all save two (both dioestrous females) showed a pressor response (Fig. 2*A*). The magnitude of the pressor response was variable, but it was usually of the order of a 10–20 mm Hg rise for a dose of 50 m-u. oxytocin, and in most cases the response lasted for 4 min or more. The reason for the lack of response after pithing in the two females was not apparent. Of the eleven rats initially showing a pressor response to oxytocin, either because they were in oestrus or had been treated with stilboestrol, seven gave an exactly equal pressor response after pithing. The remaining four showed an enhanced rise in blood pressure to similar doses of oxytocin (Fig. 2*B*). The action of vasopressin was also tested in twenty of the animals. In all but five (three oestrous and two dioestrous females) the pressor response to vasopressin

was increased. In the five exceptions the magnitude of the pressor response was similar to that seen before pithing.

In all the pithed rats the general blood pressure was between 40 and 60 mm Hg. That the appearance of the pressor response to oxytocin was not due solely to this low level was shown in two males and four dioestrous

TABLE 2. The effect of pithing on the response to oxytocin

| Effect of oxytocin on blood pressure before pithing | No. of rats | Effect of oxytocin after pithing | |
|---|---|---|---|
| | | Pressor | No effect |
| Pressor | | | |
| Oestrous females | 6 | 6 | — |
| Stilboestrol-treated males | 5 | 5 | — |
| No effect | | | |
| Dioestrous females | 8 | 6 | 2 |
| Normal males | 4 | 4 | — |

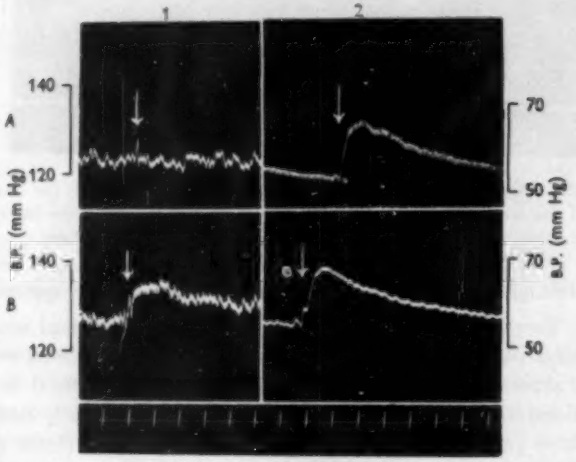



Fig. 2. The effect of 50 m-u. oxytocin on the blood pressure of (*A*) the dioestrous and (*B*) the oestrous rat, (1) before and (2) after pithing. Time marker, 30 sec.

females, to which intravenous infusions of either vasopressin or noradrenaline were given to restore the blood pressure to the levels which had been observed before pithing. In all cases oxytocin was still pressor both during and after the infusions, though no pressor effect had been present before the animals were pithed (Fig. 3).

*Vagotomy.* Bilateral cervical vagotomy was carried out during observations on a total of thirty-one rats. The results are given in Table 3. Of thirteen dioestrous females, normally showing no pressor response to

oxytocin, a pressor response appeared in six after bilateral vagotomy, the remaining seven still showing no increase in blood pressure. Of two normal males, a pressor response to oxytocin occurred in one after vagotomy. In all cases where a pressor response to oxytocin developed after section of the vagi, this was of the order of a 10–20 mm Hg rise for a dose of 50 m-u. oxytocin. In the twelve females normally showing a pressor response to

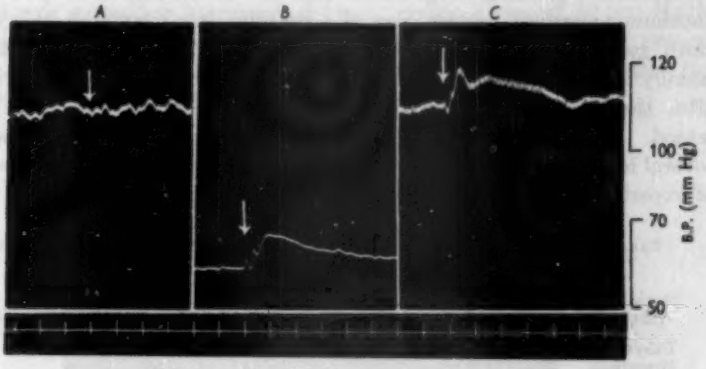



Fig. 3. The effect of 50 m-u. oxytocin on the blood pressure of the dioestrous rat, (*A*) before and (*B*) after pithing, and (*C*) during the infusion of Pitressin 0·5 m-u./min after pithing. Time marker, 30 sec.

TABLE 3. The effect of bilateral cervical vagotomy on the response of the rat to oxytocin

| Before section of vagi | Total no. of rats | Response to oxytocin after section | | |
|---|---|---|---|---|
| | | Unchanged | Increased | Decreased |
| Oxytocin pressor | | | | |
| Female oestrous or pregnant | 12 | 9 | 2 | 1 |
| Stilboestrol-treated male | 4 | 3 | 1 | — |
| Oxytocin no effect | | Pressor | No effect | |
| Female dioestrous | 13 | 6 | 7 | |
| Normal male | 2 | 1 | 1 | |

oxytocin (in oestrus or late pregnancy) this pressor response was unaltered by nerve section in nine, increased in two, and reduced in one. In four males pre-treated with stilboestrol the pressor response was unaltered in three after vagotomy, and increased in the fourth.

*Decerebration* was carried out on four female rats. Of these two were in oestrus and showed the typical pressor response to oxytocin, while the other two were dioestrous and their blood pressure was unaffected by 50 m-u. oxytocin. After decerebration the two oestrous rats continued to give an unchanged pressor response to oxytocin. The two dioestrous rats

developed a pressor response of 10–20 mm Hg following a dose of 50 m-u. oxytocin. In all four rats the response to vasopressin was increased by 50–60%.

*The effects of autonomic blocking agents*

Since crude surgical procedures such as decerebration, pithing, and vagotomy could alter the response to oxytocin, it was decided to use a variety of autonomic blocking drugs in the hope of determining which part of the autonomic nervous system was of particular importance in this effect. Both ganglionic and peripheral blocking agents were used, and were usually administered intravenously during the course of an experiment, after the normal response to posterior-lobe hormones had been determined. In some cases the blocking agents were given subcutaneously several hours before the observations were begun. The results of this series of experiments are summarized in Table 4.

TABLE 4. The effect of autonomic blocking agents on the response to oxytocin

| Blocking agent given during experiment | No. of rats | | Effect before block | | Effect after block | |
|---|---|---|---|---|---|---|
| | *F* | *M* | Pressor | No effect | Pressor | No effect |
| Dihydroergotamine | 6 | 1 | 2 | 5 | 7 | 0 |
| Bretylium | 4 | 1 | 1 | 4 | 4 | 1 |
| Dibenamine | 6 | 0 | 2 | 4 | 5 | 1 |
| TEA | 7 | 2 | 5 | 4 | 9 | 0 |
| Atropine | 10 | 3 | 4 | 9 | 4 | 9 |
| Blocking agent given before experiment | | | | | | |
| Dihydroergotamine | 4 | 0 | — | — | 4 | 0 |
| Bretylium | 4 | 0 | — | — | 4 | 0 |
| Reserpine | 6 | 2 | — | — | 8 | 0 |

*F* = female; *M* = male.

*Dihydroergotamine.* Dihydroergotamine, 0·05–0·1 mg, was injected intravenously into seven rats. After an initial abrupt fall the blood pressure always returned to slightly below the original level. That the dose given was adequate in all cases was shown by the reversal of the pressor response to injected adrenaline, and the abolition of the pressor response to tyramine.

In four dioestrous and one male rat, in which 50 m-u. oxytocin had initially no effect on the blood pressure, this dose produced a rise in blood pressure of 5–14 mm Hg in all animals after intravenous dihydroergotamine. In all cases the pressor response to vasopressin was increased by 30–50% over that previously seen (Fig. 4). For example, in one animal 0·8 m-u. vasopressin raised the blood pressure by 10 mm Hg before the block, and by 15 mm Hg after blocking. The change in the responses was apparent as soon as the general blood pressure had become steady after the administration of the blocking agent (5–15 min), and the magnitude of the responses did not vary over the period of 2–2½ hr during which they

were observed. In two other dioestrous females the effect of oxytocin on the mesenteric vessels was observed before and after dihydroergotamine. In both animals a dose of 50 m-u. oxytocin, given intravenously, was dilator to the small mesenteric vessels before blocking, and constrictor after blocking.

In two oestrous rats the pressor response to oxytocin was unaffected by the blocking agent. In both these animals the pressor response to vasopressin was somewhat increased, 0·4 m-u. giving a rise of 10 mm Hg in both animals before dihydroergotamine, and 14 and 15 mm Hg respectively after blocking. These effects persisted for at least 2 hr after the development of the block.

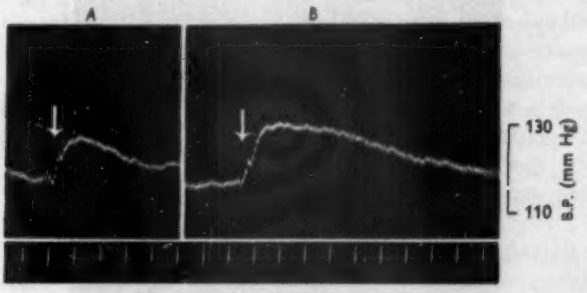



Fig. 4. The effect of 0·8 m-u. Pitressin on the blood pressure of the dioestrous rat, (*A*) before and (*B*) after 0·1 mg dihydroergotamine intravenously. Time marker, 30 sec.

In four females 0·2 mg dihydroergotamine was injected subcutaneously 2–3 hr before the start of the experiment. Of these four three were dioestrous and would not normally have been expected to give a pressor response to oxytocin, while the fourth was in an advanced stage of pregnancy, when a pressor response would normally have been obtained. In all four animals a pressor response to oxytocin was present at the time of observation, 50 m-u. raising the blood pressure by 10–15 mm Hg, and there was a depressor response to injected adrenaline, showing that the dose of dihydroergotamine had been sufficient.

*Bretylium tosylate.* Bretylium tosylate, 1–2 mg, was injected intravenously into each of five rats in which the normal responses to vasopressin and oxytocin had been established. The blood pressure responses were tested at intervals for 2–2½ hr after the administration of the blocking agent.

In three dioestrous females and one normal male oxytocin was, as expected, without effect on the blood pressure before blocking. After

bretylium the blood pressure rose 10–15 mm Hg following a dose of 50 m-u. oxytocin in two of the females and the male (Fig. 5*A*). The interval between the time of administration of the blocking agent and the appearance of the pressor response was variable, lying within the range of 5–60 min. The other female was exceptional in that no pressor response to oxytocin had developed when the experiment was concluded 2 hr after the bretylium administration. In only one animal was the magnitude of the pressor response to vasopressin increased, and in that animal the increase

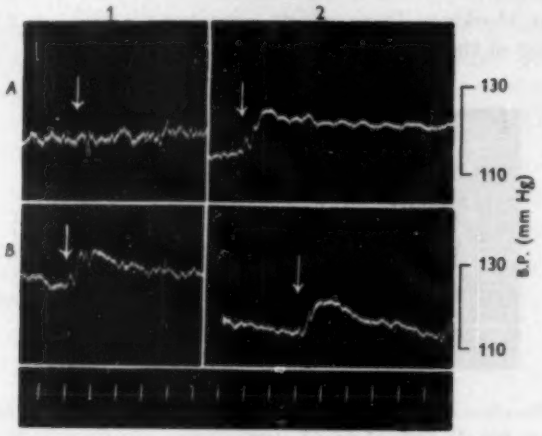



Fig. 5. The effect of 50 m-u. oxytocin on the blood pressure of (*A*) the dioestrous and (*B*) the oestrous rat, (1) before and (2) after 1 mg bretylium tosylate intravenously. Time marker, 30 sec.

was of questionable significance, since 0·8 m-u. vasopressin raised the blood pressure by 10 mm Hg before bretylium was given, and by 13 mm Hg afterwards. In the one oestrous female tested the pressor responses to both oxytocin and vasopressin were unaltered for 2 hr after bretylium was given (Fig. 5*B*), 30 m-u. oxytocin or 0·4 m-u. vasopressin raising the blood pressure by 10 mm Hg throughout the experiment.

Of four rats in which bretylium (2 mg) was injected subcutaneously 2½ hr before the start of observations, all gave pressor responses to oxytocin. Of these four, two were in the dioestrous state, one in oestrus, and one in late pregnancy. In two of these animals (one oestrous and one dioestrous) a marked response to the injection of 0·9% NaCl solution was noticed; in the former a rise in blood pressure occurred and in the latter a fall in pressure. These volume responses interfered with the assessment of the results of injection of test drugs. A similar difficulty was not

encountered in those experiments in which bretylium was given intravenously during the course of the experiments.

*Dibenamine.* Dibenamine was administered intravenously during observations on six rats, in 2 or 3 doses of 200 $\mu$g at 10 min intervals (Dekanski, 1952). In all but one animal the response to intravenous adrenaline was abolished for the remainder of the experiment.

In four dioestrous rats 50 m-u. oxytocin had, as usual, initially no pressor action. In three of these animals the administration of dibenamine caused the appearance of a pressor response to the same dose of oxytocin. In the fourth this dose was still without effect. This was the rat in which the response to adrenaline was not abolished by dibenamine; presumably the block was ineffective, though the dose was the same as that which proved adequate in the other animals. In the three rats in which a pressor response to oxytocin appeared after blocking, the pressor response to vasopressin was increased. For example, in one animal 0·6 m-u. vasopressin raised the blood pressure by 10 mm Hg before blocking, and this same degree of elevation was regularly produced by only 0·3 m-u. after blocking. In two oestrous females the rise in blood pressure of 10 mm Hg following 50 m-u. oxytocin or 0·4 m-u. vasopressin was unaltered by the administration of dibenamine.

*Tetraethylammonium iodide (TEA)* (4–8 mg) was administered intravenously to each of nine rats while the blood pressure was recorded. The immediate response to the drug was variable; a rise in blood pressure, a fall, or no change being recorded, probably depending on the size of dose and speed of injection. In four rats in which there was initially no pressor response to oxytocin (two males and two dioestrous females) the administration of TEA resulted in the appearance of a pressor response to oxytocin, though in two cases (one male and one female) this was small, 50 m-u. giving a rise of only 6 mm Hg. In all four the pressor response to vasopressin was increased. In five oestrous or stilboestrol-treated rats oxytocin was pressor both before and after TEA administration; in two out of the five the size of the pressor response to both oxytocin and vasopressin was increased, and in three out of the five the pressor responses to both hormones were unchanged in either magnitude or duration.

*Atropine.* Atropine was injected intravenously into each of thirteen rats, the dose used (1–2 mg) being sufficient to block all depressor responses to injected acetylcholine. In six dioestrous females and three normal males oxytocin had no effect on the blood pressure either before or after atropine administration, while the pressor response to vasopressin remained unchanged. In four oestrous females the pressor response to both oxytocin and vasopresin was unaltered by atropine. It was also noted that such treatment with atropine did not prevent the development of a pressor

response to oxytocin if sympathetic blocking agents were later administered (five rats).

*Reserpine.* Since Burn & Rand (1958) have shown that in certain circumstances blood vessels which have lost their stores of noradrenaline are unable to contract normally, it was decided to see whether pre-treatment with reserpine would prevent the development of a pressor response to oxytocin. Eight rats (six female and two male) were treated with 0·5 mg reserpine subcutaneously daily for 2–4 days. On the day following cessation of treatment the response to oxytocin was tested. In all the animals oxytocin had a pressor action, 50 m-u. raising the blood pressure by 10–25 mm Hg, though all females showed dioestrous vaginal smears

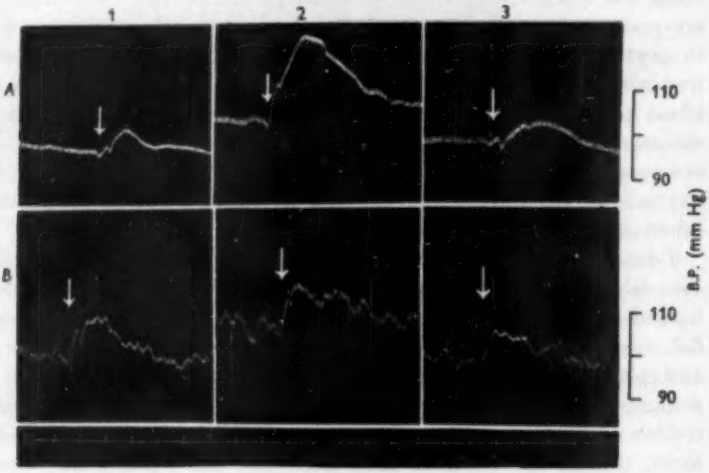



Fig. 6. The effect of (*A*) 0·02 mg tyramine, and (*B*) 50 m-u. oxytocin on the blood pressure of the reserpine-treated rat. (1) Before infusion of noradrenaline, (2) during infusion of noradrenaline 0·05 μg/min, (3) 30 min after end of infusion. Rats treated with two consecutive daily doses of 0·5 mg reserpine subcutaneously. Time marker, 30 sec.

during administration of reserpine and on the day on which the blood pressure was observed. The intravenous infusion of noradrenaline at rates of 0·05–2·0 μg/min for 30–40 min did not alter the pressor response to oxytocin (Fig. 6*B*), though it did increase the pressor response to tyramine (Fig. 6*A*). In all the animals the pressor response to vasopressin tended to be greater than in normal dioestrous animals, e.g. 0·4–0·5 m-u. raised the blood pressure by 10 mm Hg, as compared with an average of 0·6–0·8 m-u. necessary for inducing the same degree of raise in the normal dioestrous rat. Two female rats were treated with reserpine for two days, and

stilboestrol (3·5 μg/100 g) given subcutaneously on the second day. On the following day, when observations were made, it was found that oxytocin was pressor in both, 50 m-u. raising the blood pressure by 10 and 13 mm Hg respectively; that is, stilboestrol and reserpine showed no mutual interference.

*Infusion of dilator agents during sympathetic blockade*

Since it was found, as described above, that the infusion of vasodilator substances to dioestrous rats did not cause the appearance of a pressor response to oxytocin, though dilatation and fall in blood pressure induced by sympathetic blocking agents did so, it was of interest to determine whether the infusion of dilators after sympathetic blockade would affect

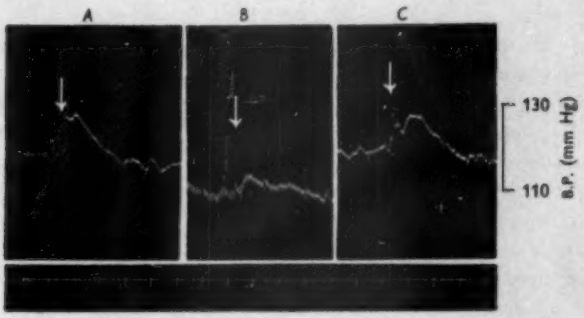



Fig. 7. The effect of 50 m-u. oxytocin on the blood pressure of the dioestrous rat after 0·1 mg dihydroergotamine intravenously. (*A*) Before infusion of isoprenaline, (*B*) during infusion of isoprenaline 0·02 μg/min, and (*C*) after infusion of isoprenaline. Time marker, 30 sec.

the pressor action of oxytocin. Six rats were given infusions of isoprenaline or acetylcholine after sympathetic blockade with bretylium or dihydroergotamine, and the effects of oxytocin and vasopressin tested before, during and after the infusions. In five animals the pressor response to oxytocin was reduced during the infusions, with recovery of the normal pressor response after the infusion had ended and the blood pressure had returned to its previous level (Fig. 7). In one animal the response to oxytocin was completely abolished during the infusion of isoprenaline after the administration of dihydroergotamine, but was again present after the infusion ended. The pressor response to vasopressin was similarly reduced during the infusions in all these experiments.

## DISCUSSION

Previous results have been regularly confirmed, that oxytocin is a vasodilator without effect on blood pressure in dioestrous female and in male rats, whereas it is constrictor and pressor in oestrogen-treated dioestrous females, oestrous females, in late pregnancy, and in males treated with oestrogen with or without the addition of progesterone (Lloyd, 1959*a*, *b*). The present experiments have further shown two things. First, that a low blood pressure and dilated peripheral vasculature do not of themselves cause small doses of oxytocin to become pressor and constrictor, since without exception oxytocin did not raise the blood pressure of dioestrous or male rats during the infusion of a variety of vasodilator agents. Nor when the low blood pressure of pithed rats was raised with vasoconstrictors did oxytocin act as a vasodilator. It is, however, possible to reduce or abolish the constrictor response to both oxytocin and vasopressin by extreme vasodilatation, such as can be produced by the infusion of a vasodilator after the administration of a sympathetic blocking agent. Secondly, it has been shown that the nervous system plays an important role in the maintenance of the dilator response to oxytocin. Since atropine did not alter the existing response to oxytocin, whatever that response might be, and did not prevent a change in response induced by other means, it is concluded that cholinergic nerves are not involved. On the other hand, interference with other parts of the autonomic nervous system always converted a dilator action of oxytocin to a pressor one, whilst leaving untouched an already developed pressor response. Thus, surgical methods (pithing and decerebration) which left the post-ganglionic neurones intact caused oxytocin to become a pressor substance. That decerebration as well as pithing was effective suggests the importance of higher centres in the nervous system. Bilateral cervical vagotomy was sometimes effective, probably because in the rat it is difficult to divide the vagus nerves without damage to cervical sympathetic fibres. Drugs were used to block different parts of the peripheral autonomic nervous system in an attempt to determine the pathways of the central control. Again, oxytocin became constrictor following the administration of any sympathetic blocking agent, whether the block lay in ganglia or in a part or the whole of the sympathetic nervous system. It was interesting that reserpine caused oxytocin to become a pressor substance and vasopressin to be somewhat more active than in normal dioestrous rats. Thus, even this type of interference with the sympathetic nervous system brought out the constrictor property of oxytocin. That these varied peripherally-acting drugs, with presumably little or no central action, convert the dilator action of oxytocin to a constrictor one suggests that whatever influence the central

nervous system exerts is transmitted directly through the peripheral nerves, and is not dependent on any unknown indirect neurohumoral path. For the present, the following suggestion is put forward to account for the facts; that oxytocin, though feeble in its action, like vasopressin contracts the smooth muscle of blood vessels, but that with an intact sympathetic nervous system a diminution of constrictor tone more than compensates for the direct vasoconstriction, unless large doses of oxytocin are used (van Dyke, Adamsons & Engel, 1955). This means one of two things: that intravenously administered oxytocin has a central indirect as well as a peripheral direct action on the vascular system, or that oxytocin given intravenously allows time for centrally initiated homoeostasis. Little information is at present available to permit a choice between these possibilities, except that after oxytocin homoeostasis is curiously and imperfectly achieved in that small blood vessels can be shown to dilate in both rat and man, and in man blood pressure is maintained by an increase in cardiac output (Kitchin, Lloyd & Pickford, 1959). This perhaps points to a central action of oxytocin.

Another interesting fact is the similarity found between the results of administration of oestrogens and of denervation, whether surgical or chemical. Probably the peripheral vasodilator action of oestrogens is not the cause of the change in response to oxytocin following ovarian hormone treatment, since other dilator agents do not induce the appearance of such a change. The possibility must therefore be considered that oestrogens can depress sympathetic nervous activity or block the hypothetical central dilator action of oxytocin, thus unmasking the peripheral constrictor effect. Further experiments are needed to investigate this possibility and its implications.

SUMMARY

1. The blood pressure responses of rats to oxytocin and vasopressin were studied during the infusion of vasodilator substances, and after surgical or chemical interruption of autonomic nervous pathways.

2. None of the vasodilators tested altered the responses to the posterior-lobe hormones.

3. On ten out of twelve occasions following pithing or decerebration in the male or dioestrous rat oxytocin, which normally did not affect the blood pressure, acted as a pressor substance. The pressor response persisted even when the blood pressure was raised by infusion of vasoconstrictor substances. In most instances the pressor response to oxytocin was accompanied by an increased sensitivity to the pressor action of vasopressin.

4. Similar changes in response were seen after ganglionic or peripheral sympathetic blockade, but not after atropine.

5. It is suggested that the smooth muscle of the vascular system is caused to contract by a direct action of oxytocin, and that the absence of any change in blood pressure and the vasodilatation seen in dioestrous and male rats is due to an overriding central activity.

6. The similarity of action of oestrogens and of sympathetic blockade on the responses of the rat to oxytocin are discussed.

Our thanks are due to Professor V. du Vigneaud for a gift of highly purified oxytocic extract of posterior pituitary; to Dr D. A. Long of the Wellcome Foundation Ltd for a gift of Darenthin, and to Abbott Laboratories for serotonin; also to the United States Air Force (contract AF61 (052)-272) for financing this research.

## REFERENCES

BROOKS, F. P. & PICKFORD, M. (1958). The effect of posterior pituitary hormones on the excretion of electrolytes, in dogs. *J. Physiol.* **142**, 468–493.

BURN, J. H. & RAND, M. J. (1958). The action of sympathomimetic amines in animals treated with reserpine. *J. Physiol.* **144**, 314–336.

DEKANSKI, J. (1952). The quantitative assay of vasopressin. *Brit. J. Pharmacol.* **7**, 567–572.

KITCHIN, A. H., LLOYD, S. & PICKFORD, M. (1959). Some actions of oxytocin on the cardiovascular system in man. *Clin. Sci.* **18**, 399–406.

LLOYD, S. (1959*a*). The vascular responses of the rat during the reproductive cycle. *J. Physiol.* **148**, 625–632.

LLOYD, S. (1959*b*). Changes in the vascular responses of the rat during pregnancy. *J. Physiol.* **149**, 586–592.

NIXON, W. C. W. & SMYTH, C. N. (1957). Physiological and clinical aspects of uterine action. *J. Obstet. Gynaec. Brit. Emp.* **64**, 35–46.

VAN DYKE, H. B., ADAMSONS, K. & ENGEL, S. L. (1955). Aspects of the biochemistry and physiology of neurohypophysial hormones. *Recent Progr. Hormone Res.* **11**, 1–41.

*J. Physiol.* (1961), **155**, *pp.* 175–186
*With* 1 *plate and* 6 *text-figures*
*Printed in Great Britain*



# ON THE NATURE OF VIBRATION RECEPTORS IN THE HIND LIMB OF THE CAT

By C. C. HUNT

*From the Department of Physiology, University of Utah College of Medicine, Salt Lake City, Utah, U.S.A.*

(*Received* 21 *September* 1960)

Hunt & McIntyre (1960) recently investigated the conduction velocity and receptor characteristics of afferent fibres in the nerves to flexor longus digitorum (FLD) and to the associated interosseous region of the cat. A considerable number of fibres in this interosseous nerve showed discharge to vibratory stimuli applied to the tibia or fibula. The receptors of many such fibres were extremely sensitive, responding to remotely induced vibration such as that produced by slight jarring of the floor or by light stroking of the skin of the lower limb.

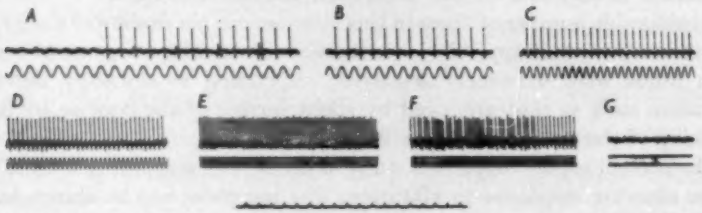
The present report concerns a study of the type of receptor related to the fibres showing responses to vibration. The receptors will be shown to be Pacinian corpuscles. A large number of such corpuscles lie, in close association with the periosteum, along the tibia, near the interosseous membrane. Evidence will be presented suggesting that Pacinian corpuscles are concerned with vibration sensibility.

## METHODS

Adult cats were anaesthetized with sodium pentobarbital (Nembutal; Abbott Laboratories). The region from popliteal fossa to calcaneus was exposed by a longitudinal incision and the gastrocnemii, soleus and plantaris muscles were removed. The interosseous nerve was identified and traced distally. It courses between the medial and lateral heads of FLD, then through the lateral head of FLD, to run alongside the tibia just dorsal to the interosseous membrane. In order fully to expose the interosseous nerve and the interosseous membrane, the lateral head of FLD was carefully removed. This stage of the dissection was carried out with a stereomicroscope, at a magnification of 7 to 20 times. Exposed tissues were covered by a pool of paraffin oil. Afferent discharge was recorded from Pacinian corpuscles in the following ways: from fibres in the interosseous nerve, from dorsal root filaments containing single fibres from the interosseous nerve, or from nerve fibres from individual corpuscles. In certain experiments afferent discharge was recorded from the sural nerve or the muscle nerve to the medial head of FLD. For recording, fine platinum-iridium electrodes were used, the potentials being amplified and displayed on a Tektronix 502 oscilloscope. Vibration was provided by an earphone with a glass stylus attached to the diaphragm; this was driven by a sine-wave oscillator of variable frequency and amplitude.

RESULTS

Exposure of the interosseous nerve along the lateral surface of the tibia revealed a large number of translucent corpuscles. Nearly all were bound by connective tissue to the tibia but some were occasionally found lying in muscle. More distally, near the ankle joint, a cluster of six to eight corpuscles was usually found, some of which abutted against the lower surface of the fibula. The corpuscles appeared to be more intimately bound to the periosteum than to the interosseous membrane, although some connective tissue strands were present between the latter and some of the corpuscles. No detailed measurements of the number or the dimensions of these corpuscles have yet been made. However, it is estimated that there may be 40–50 associated with the interosseous nerve. They are very similar in appearance to the Pacinian corpuscles in the mesentery, and at least as




Text-fig. 1. Discharge, in response to vibration, recorded from single afferent fibre from a Pacinian corpuscle located adjacent to the tibia. Upper trace, nerve impulses; lower trace, frequency of vibration applied to corpuscle. Final frequencies (c/s): *A* 90, *B* 102, *C* 180, *D* 550, *E* 650, *F* 700, *G* 1000. Time marker, 10 msec.

large. A photograph of the corpuscles *in situ* is shown in Pl. 1 *A*. Some can be seen to be closely bound to the tibial periosteum. Such corpuscles could be dissected away from the periosteum by incision of a connective tissue envelope. A number of these corpuscles were excised and fixed in osmium tetroxide (1%). On cross-section, the typical laminar structure and central axon of Pacinian corpuscles were seen, their appearance being similar to mesenteric corpuscles (Quilliam & Sato, 1955, and others).

Several corpuscles were isolated, together with their afferent nerve fibres, and completely freed from surrounding tissue. The response of one such isolated corpuscle to vibratory mechanical stimulation is shown in Text-fig. 1. Even large-amplitude vibration of less than 85 c/s failed to evoke discharge. However, as the frequency of the sinusoidal vibration was increased, afferent impulses were found to follow each cycle of vibration in a 1:1 ratio over a frequency range from 90 to 600 c/s. Since this is the same frequency response as that found by Hunt & McIntyre (1960) when recording from afferent fibres of vibration receptors from the same region,

the latter may be identified as Pacinian corpuscles. In a number of corpuscles from the interosseous region the lower limit of effective frequency, at the amplitude of vibration used, varied between 40 and 90 c/s, the higher limit between 600 and 800 c/s. A similar range of effective frequency is noted when recording from the entire interosseous nerve and applying vibration of variable frequency to the tibia (see Text-fig. 2). The ineffectiveness of low frequencies of mechanical vibration can be attributed to the rapid adaptation of these receptors and the need for a critical rate of change of movement for generation of an impulse (Gray & Matthews, 1951*b*).

*Phase relations and recruitment.* The discharge recorded from the interosseous nerve in response to mechanical vibration applied to the tibia or fibula shows a considerable temporal dispersion of action potentials associated with each cycle of vibration. Text-figure 2 shows the response recorded from an interosseous nerve to mechanical vibration of bone at several frequencies. It may be noted that the dispersion becomes less as the frequency is increased. A clearer view of the phase difference in the responses of corpuscles to mechanical vibration may be seen by recording from a branch of the interosseous nerve supplying only a few corpuscles. Text-figure 3 illustrates the response recorded from such a branch when vibration at 200 c/s of varied amplitude was applied to nearby tissue. As the amplitude of vibration was increased, at the same frequency, units were recruited showing different temporal relations to the driving sine wave. Even when the amplitude of vibration was considerably above threshold, phase differences in the several units persisted. Such phase differences probably depend upon mechanical factors in transmission of vibration to the several corpuscles involved as well as threshold differences.

*Sensitivity.* The extreme sensitivity of some vibration receptors has already been noted by Hunt & McIntyre (1960). Further observations on this aspect of the behaviour of these receptors were made in the course of the present study. In some fibres from Pacinian corpuscles apparently random discharges were noted in the absence of any applied stimulation. Recording at high gain from a sensitive microphone placed upon the experimental table revealed that each discharge from the receptor was associated with a slight, but detectable, shaking of the table transmitted through the floor (Text-fig. 4). Another interesting example of the marked sensitivity of some Pacinian corpuscles is provided by the following observation. A corpuscle was being driven at a frequency of about 500 c/s by a glass stylus attached to the electromechanical transducer, and the impulses, which occurred at the frequency of vibration, were monitored by a loudspeaker. When the sine-wave oscillator driving the transducer was turned off the discharge continued at the same frequency, but stopped

abruptly when the output of the audio monitor was reduced. The sound associated with each impulse caused sufficient vibration to continue the discharge after driving of the transducer by the sine-wave oscillator was discontinued.

*Vibration receptors in skin.* In view of the role generally attributed to cutaneous receptors in vibration sensibility (see Discussion), the response to vibratory stimuli applied to the skin was studied by recording from cutaneous afferent fibres. In the experiment illustrated in Text-fig. 5, recording electrodes were placed on the distal end of the cut main branch

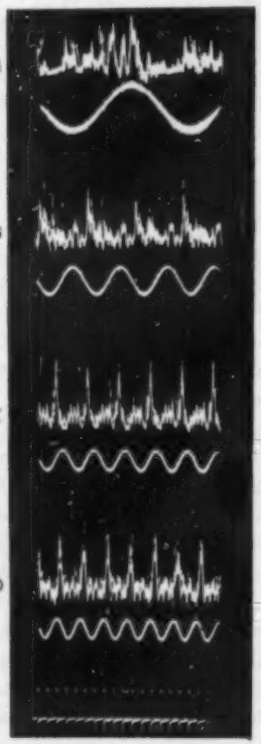



Fig. 2

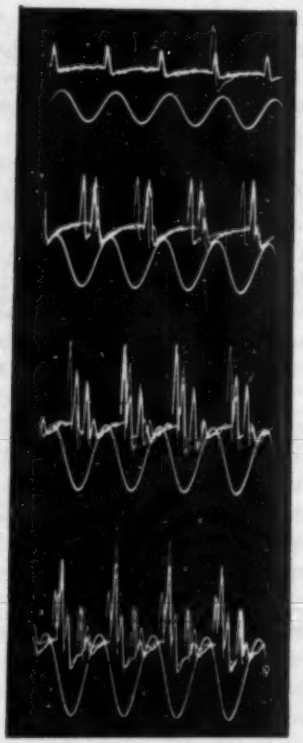
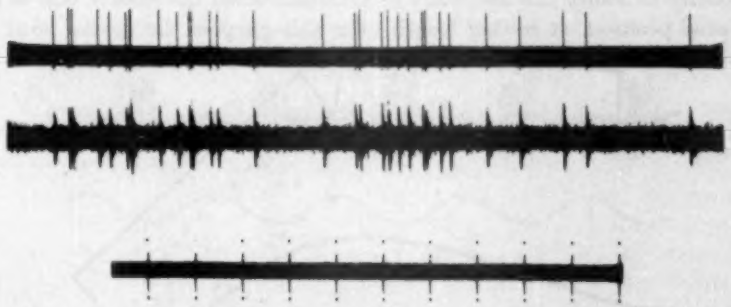


Fig. 3

Text-fig. 2. Discharge recorded from entire interosseous nerve. Each record shows superimposition of several sweeps. Lower trace shows frequency of vibration (c/s): *A* 80, *B* 100, *C* 300, *D* 400. Time marker, 1 msec.

Text-fig. 3. Recording from nerve branch to several Pacinian corpuscles. Amplitude of vibration at 200 c/s increased from above downward, indicated on lower trace. Note recruitment of units.

of the sural nerve. This particular nerve innervated a region enclosed by the interrupted line. A stylus, attached to the electrical-mechanical transducer, was used to explore this region of skin point by point. Large-amplitude vibration at a frequency of 400 c/s applied anywhere in this area elicited discharge in the sural nerve which showed a 1:1 relationship to the driving frequency. However, when the amplitude of vibration was reduced sufficiently, a discharge in tempo with the frequency of vibration could be detected only at three points, marked *a*, *b* and *c*. Regions away



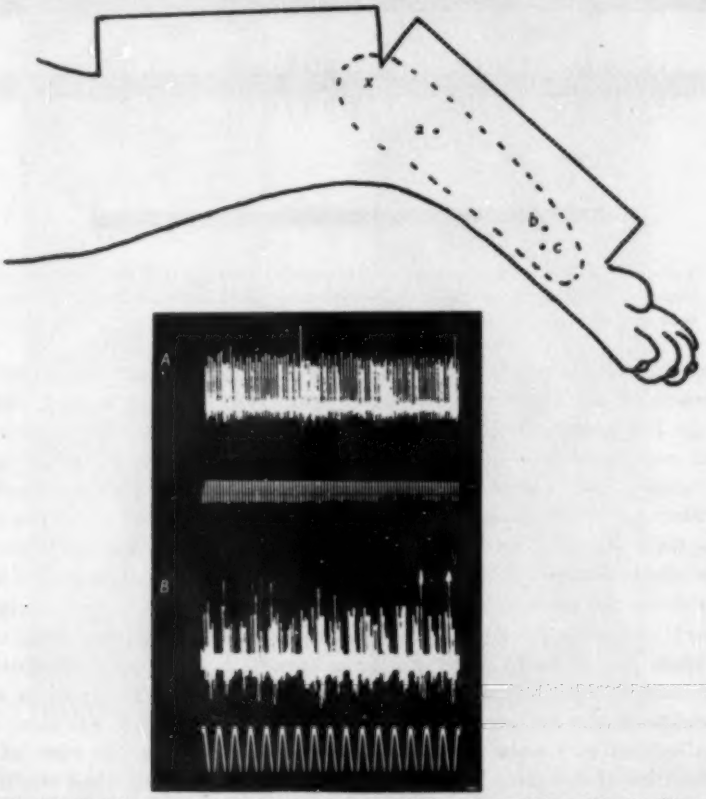
Text-fig. 4. Upper trace; background discharge from a single fibre from a Pacinian corpuscle. Middle trace; vibration recorded by sensitive microphone. Below, time marker, 1 sec.

from these three points showed no such response to either 200 or 400 c/s vibration of low amplitude. However, when the frequency was reduced to 40 c/s, low amplitude vibration caused a discharge of impulses associated with each cycle from any position of the innervated area (Text-fig. 5).

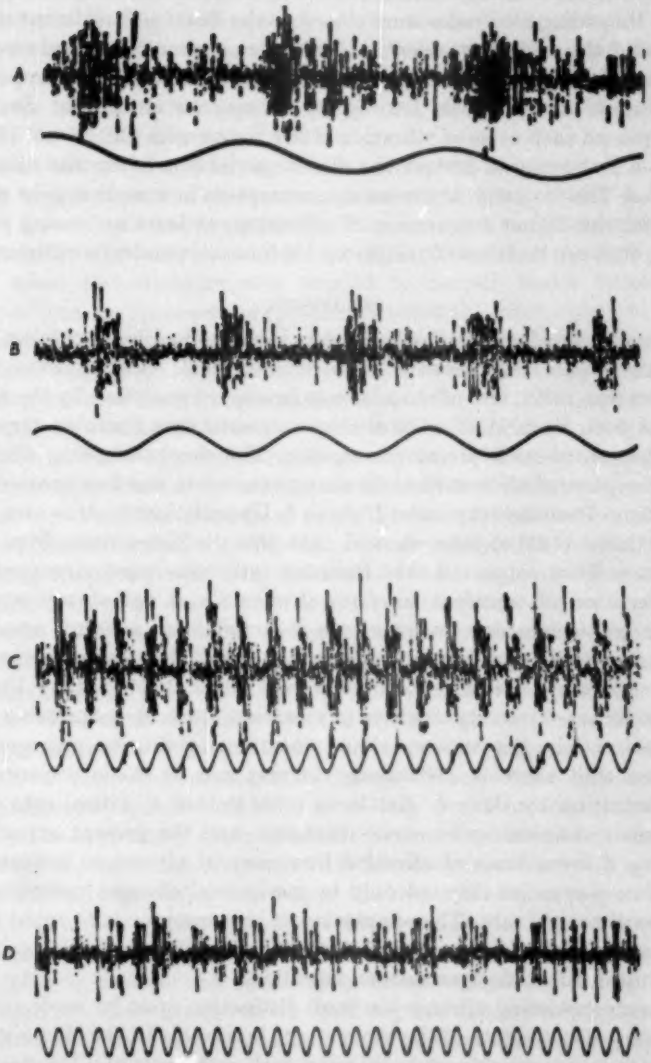
Points *a*, *b* and *c* were marked with Indian ink, after which a longitudinal incision was made medial to the border of the innervated area. The skin was then dissected free from underlying tissues and reflected laterally. Needles were inserted through the points *a*, *b* and *c* until their tips were visible on the inner surface of the skin. With a dissecting microscope a search was made for Pacinian corpuscles over the entire innervated area. In close proximity to point *a* a large corpuscle was found. Similarly a corpuscle was closely adjacent to point *b*, and at *c* two corpuscles were found to lie side by side (Pl. 1 *B*). Only one other corpuscle was seen; this was located at a point several millimetres medial to *a*. In view of the difficulties of detecting responses in single afferent fibres when recording from the whole sural nerve the failure to detect this focus of sensitivity to vibration at the site of one corpuscle is not surprising. The close correspondence between the foci of vibration sensitivity at points *a*, *b* and *c*, and the presence of Pacinian corpuscles at these sites suggests that reception of

vibration in the range from 200 to 400 c/s may be conveyed exclusively in the skin of cat by such corpuscles.

*Responses from muscle.* Muscle stretch receptors were found by Echlin & Fessard (1938) to respond to vibration when the muscle was stretched. Kuffler, Hunt & Quilliam (1951) noted that discharge from muscle spindle receptors could follow the frequency of a tuning fork (112 c/s) placed against the belly of a muscle under tension. The responses to vibration of receptors in muscle have been re-examined with a view to determine their capacity to follow the frequency of vibration when the muscle was in its normal position, at resting length. For this purpose the medial head of




Text-fig. 5. Above: distribution of foci from which discharge at 200 or 400 c/s to low-amplitude vibration could be detected in sural nerve (*a*, *b* and *c*). Below: *A*, Upper record, discharge recorded in sural nerve to sinusoidal vibration at 200 c/s, applied to point *a*; lower trace, frequency of vibration. *B*, Upper trace, discharge recorded in sural nerve to vibration at 40 c/s to point *X*; lower trace, frequency of vibration.




**Text-fig. 6. Responses of muscle receptors to vibration. Stylus vibrating at 20 (*A*), 32 (*B*), 137 (*C*), and 200 (*D*) c/s applied to belly of FLD (medial head). Upper traces, discharge recorded in distal end of cut nerve to FLD; lower traces, frequency of vibration.**

FLD was used, with its tendons left intact and the foot suspended freely in air. Recording electrodes were placed on the distal end of the cut muscle nerve and the stylus, attached to the electromechanical transducer, was placed against the belly of the muscle. The responses to varying frequencies of vibration are shown in Text-fig. 6. It may be noted that discharge accompanied each cycle of vibration at low frequencies (20, 32 and 137 c/s) but at a frequency of 200 c/s the discharge did not follow the vibratory stimulus. The majority of the mechanoreceptors in muscle appear unable to signal the higher frequencies of vibration, at least at resting muscle length, that can be followed readily by Pacinian corpuscles (see Discussion).

## DISCUSSION

Although the Pacinian corpuscle has been studied by a number of investigators (Alvarez-Bullya & de Arellano, 1953; Gray & Sato, 1953; Loewenstein, 1958, and others), its role in sensory reception by the animal has not been clarified. Earlier studies suggested that Pacinian corpuscles were concerned with pressure reception. The slowly-adapting discharge from receptors of afferent fibres in the plantar nerve was first considered to come from Pacinian corpuscles (Adrian & Umrath, 1929). However, Gray & Matthews (1951*a*) later showed that this discharge came from other receptors. They suggested that Pacinian corpuscles were concerned with the reception of transient mechanical events and not steady pressure. Discharge to constant pressure was recorded from isolated mesenteric corpuscles by Scott (1949) but this author found the corpuscle was much more sensitive to vibratory stimuli (Scott, 1949, 1951). Many Pacinian corpuscles are extremely sensitive to vibration, and a stylus pressing on the corpuscle might transmit spurious vibrations, giving an erroneous impression that there is a discharge in response to static pressure. The demonstration by Gray & Matthews (1951*b*) that a critical rate of displacement is necessary to evoke discharge, and the present experiments showing a lower limit of effective frequency of vibration, indicate that Pacinian corpuscles respond only to mechanical changes occurring at a sufficiently rapid rate. The adaptation of this receptor is so rapid that it cannot signal static pressure. It is, however, well suited to signalling of rapid mechanical changes such as vibration.

When considering vibratory stimuli distinction must be made as to the frequency components of the movements involved. In the skin a number of receptors are capable of discharging with each cycle of a low-frequency sinuosidal vibration. To vibrations below 60 c/s most Pacinian corpuscles do not discharge, although both hair and touch receptors may discharge with each cycle of movement. As frequency is raised above 60–70 c/s, discharge is evoked from Pacinian corpuscles as well as from other receptors,

but to vibration exceeding about 150 c/s the only known receptor in skin capable of following the frequency of vibration is the Pacinian corpuscle. In the region of skin innervated by the sural nerve in the cat these corpuscles are few in number and dispersed. The location of such corpuscles has been shown to correspond to the only foci of sensitivity to low-amplitude vibration at 200 or 400 c/s.

Muscle stretch receptors are known to respond to vibration, but they follow high frequencies only under critical conditions. Echlin & Fessard (1938) found that vibratory stimuli at frequencies up to 530 c/s could evoke discharge in a muscle nerve of the cat at the frequency of vibration, but only when the vibration was applied to muscle under considerable external tension. These authors pointed out that the discharge from stretch receptors often occurred at some submultiple of the frequency of vibration and that alternation of discharge probably occurred in the multifibre discharge recorded from whole muscle nerves at the higher frequencies. Thus, discharge in a multi-fibre nerve which follows the highest frequency of vibration may not represent individual receptor discharge at that rate. This is supported by Pfaffman's (1939) observations on vibration responses from receptors in teeth recorded from single fibres and whole nerve. Echlin & Fessard (1938) observed that driving of the stretch receptor discharge at the vibrating frequency was most readily obtained when the receptor was already discharging, due to stretch, at a frequency near that of the applied vibration. Further, stretch receptors of muscles under low tension responded only to much lower frequencies of vibration, a fact confirmed in the present study. The stretch receptors concerned were probably muscle spindles (Kuffler *et al.* 1951). With a critical degree of stretch of such endings, sinusoidal vibration applied to the muscle may be expected to produce a recurrent mechanical change wherein the increment in deformation associated with each cycle may evoke an impulse. Similar considerations probably apply to a variety of mechanoreceptors. However, the range of frequencies that can be followed by muscle stretch receptors appears to be considerably less than in the case of Pacinian corpuscles and depends to a very great extent upon the presence of adequate stretch in the muscle. For these reasons alone muscle stretch receptors seem unlikely candidates for a major role in vibration reception by deep tissues.

The present study has shown a large group of Pacinian corpuscles closely associated with the tibial periosteum. Their afferent fibres course centrally by way of the interosseous nerve branch associated with the FLD nerves. The responses recorded from these corpuscles are identical to those found in interosseous nerve fibres from vibration receptors (Hunt & McIntyre, 1960). Such corpuscles are strategically located for sensing bone-transmitted vibration. The sensitivity of many of these corpuscles is so great

that very small vibrations transmitted through the skin and soft tissues readily evoke discharge. Pacinian corpuscles have also been found adjacent to tendons (Adrian & Umrath, 1929; Gray & Matthews, 1951*a*), near joints (Skoglund, 1956), and in muscle (Hinsey, 1934). All such deeply located Pacinian corpuscles could play a significant role in the reception of vibration transmitted through the skeletal system. The Pacinian corpuscles have been shown to follow vibratory stimuli up to a frequency of about 700–800 c/s. Discharge, intermittent with respect to each cycle of vibration, may occur up to 1000 c/s, but above this frequency no discharge is elicited. Of the known deep receptors in the limb the only one capable of responding to such high frequencies of vibration are the Pacinian corpuscles.

The literature on vibration sensation in man is extensive and conflicting (for a review see Geldard, 1940) and cannot be considered in detail here. From the standpoint of possible receptor mechanism, the data from the cat suggest the Pacinian corpuscle as the most probable receptor, certainly if one considers frequencies in excess of 200 c/s. The location of such corpuscles in both skin and deep structures would explain both cutaneous and bone-transmitted vibration sensibility. The upper frequency limit of vibration sensibility in man is not clear. Newman, Doupe & Wilkins (1939) place it at 600–1000 c/s, dependent on location. This corresponds to the upper limit of the frequency range of Pacinian corpuscles. However, others have placed the upper limit of vibration sensibility in man at 10,000 c/s or more (Geldard, 1940).

In man, foci of minimal threshold for vibration sensibility and points of sensitivity to pressure are said to coincide on the skin (Geldard, 1940). This could not be readily explained by assuming the Pacinian corpuscle is the responsible receptor. Clearly, the characteristics of Pacinian corpuscles, as observed in the cat, would not permit this receptor to subserve reception of both vibration and static pressure.

The frequency of vibration applied to skin or over bone in man can be sensed but the extent of frequency discrimination does not appear to have been studied. It is of interest to compare the probable peripheral mechanisms of frequency discrimination by the auditory system to a vibration-sensitive system that might be subserved by Pacinian corpuscles. In the former case, topographical representation of afferent fibres in the cochlea is an important feature. The relative discharge in fibres from adjacent regions of the cochlea provides significant information as to pitch, certainly at high frequencies of sound (Davis, 1957), although with sound of low frequency and high intensity the discharge frequency in individual afferent fibres may be important (Katsuki, Sumi, Uchiyana & Watanabe, 1958). In the case of vibration sensibility such as might be subserved by Pacinian corpuscles, the detection of frequency probably depends exclu-

sively on the discharge frequency in afferent fibres from such receptors. Intensity might be signalled by the number of such fibres that are transmitting signals at the frequency of vibration.

The significance of a sensory system extremely sensitive to vibration can only be surmised. Any mechanical event producing movement with components of sufficiently high frequency may be expected to evoke discharge in Pacinian corpuscles. This would occur in a wide variety of body actions such as accompany locomotion or when a limb makes contact with some object. This system is also sufficiently sensitive to allow the animal to sense very slight vibrations from external sources that are transmitted through the medium on which the animal is supported. The general role of such a sensing mechanism remains to be explored. The great sensitivity of Pacinian corpuscles to rapid mechanical stimuli, even when applied at a considerable distance, makes it necessary to consider the possible contribution of these receptors in a wide variety of experiments in which selective afferent input is sought.


## SUMMARY

1. In an investigation of receptors sensitive to mechanical vibration in the hind limb of the cat a large number of Pacinian corpuscles have been found in close relation to the tibial periosteum. These corpuscles are often extremely sensitive to rapid mechanical stimuli. They respond to sinusoidal vibration in the range from about 50 to 800 c/s. Their behaviour is similar to that previously recorded in afferent fibres from vibration receptors in the interosseous nerve.

2. Recording from afferent fibres from a number of tibial Pacinian corpuscles indicates that the impulses from different corpuscles vary in their phase relations to the sinusoidal vibration. The range of vibration frequency is generally similar. Increasing intensity of stimulation recruits additional receptors.

3. Low-amplitude vibration at 200 or 400 c/s applied to the skin evoked discharge at the frequency of vibration in the sural nerve only from discrete foci. Pacinian corpuscles were found to be located in close proximity to such foci. Vibration at lower frequency (40 c/s) evoked discharge from other cutaneous receptors.

4. Response of muscle receptors to vibration was re-examined. With muscle at resting length discharge accompanied each cycle of vibration at lower frequencies (up to about 150 c/s) but failed to do so at higher frequencies (200 c/s and above).

5. These results suggest that the only known receptor in the hind limb, in both skin and deep structures, that is capable of following high-

frequency vibration is the Pacinian corpuscle. The possible relation to vibration sensibility is discussed.

This work was supported by a research grant (B 1320) from the National Institutes of Health, Public Health Service.

## REFERENCES

ADRIAN, E. D. & UMRATH, K. (1929). The impulse discharge from the Pacinian corpuscle. *J. Physiol.* **68**, 139–154.

ALVAREZ-BULLYA, R. & DE ARELLANO, R. (1953). Local responses in Pacinian corpuscles. *Amer. J. Physiol.* **172**, 237–244.

DAVIS, H. (1957). Biophysics and physiology of the inner ear. *Physiol. Rev.* **37**, 1–49.

ECHLIN, F. & FESSARD, A. (1938). Synchronized impulse discharges from receptors in the deep tissues in response to a vibrating stimulus. *J. Physiol.* **93**, 312–334.

GELDARD, F. A. (1940). The perception of mechanical vibration. *J. gen. Psychol.* **22**, 243–308.

GRAY, J. A. B. & MATTHEWS, P. B. C. (1951*a*). Response of Pacinian corpuscles in the cat's toe. *J. Physiol.* **113**, 475–482.

GRAY, J. A. B. & MATTHEWS, P. B. C. (1951*b*). A comparison of the adaptation of the Pacinian corpuscle with the accommodation of its own axon. *J. Physiol.* **114**, 454–464.

GRAY, J. A. B. & SATO, M. (1953). Properties of the receptor potential in Pacinian corpuscles. *J. Physiol.* **122**, 610–636.

HINSEY, J. C. (1934). The innervation of skeletal muscle. *Physiol. Rev.* **14**, 514–585.

HUNT, C. C. & MCINTYRE, A. K. (1960). Characteristics of responses from receptors from flexor longus digitorum and the adjoining interosseous region of the cat. *J. Physiol.* **153**, 74–87.

KATSUKI, Y., SUMI, T., UCHIYAMA, H. & WATANABE, T. (1958). Electrical responses of auditory neurons in cat to sound stimulation. *J. Neurophysiol.* **21**, 569–588.

KUFFLER, S. W., HUNT, C. C. & QUILLIAM, J. P. (1951). Function of medullated small-nerve fibers in mammalian ventral roots: efferent muscle spindle innervation. *J. Neurophysiol.* **14**, 29–54.

LOEWENSTEIN, W. R. (1958). Generator processes of repetitive activity in a Pacinian corpuscle. *J. gen. Physiol.* **41**, 825–845.

NEWMAN, H. W., DOUPE, J. & WILKINS, R. W. (1939). Some observations on the nature of vibratory sensibility. *Brain*, **62**, 31–40.

PFAFFMAN, C. (1939). Afferent impulses from the teeth resulting from a vibratory stimulus. *J. Physiol.* **97**, 220–232.

QUILLIAM, T. A. & SATO, M. (1955). The distribution of myelin on nerve fibres from Pacinian corpuscles. *J. Physiol.* **129**, 167–176.

SCOTT, D. (1949). Response of individual Pacinian corpuscles to external stimulation. *Fed. Proc.* **8**, 142.

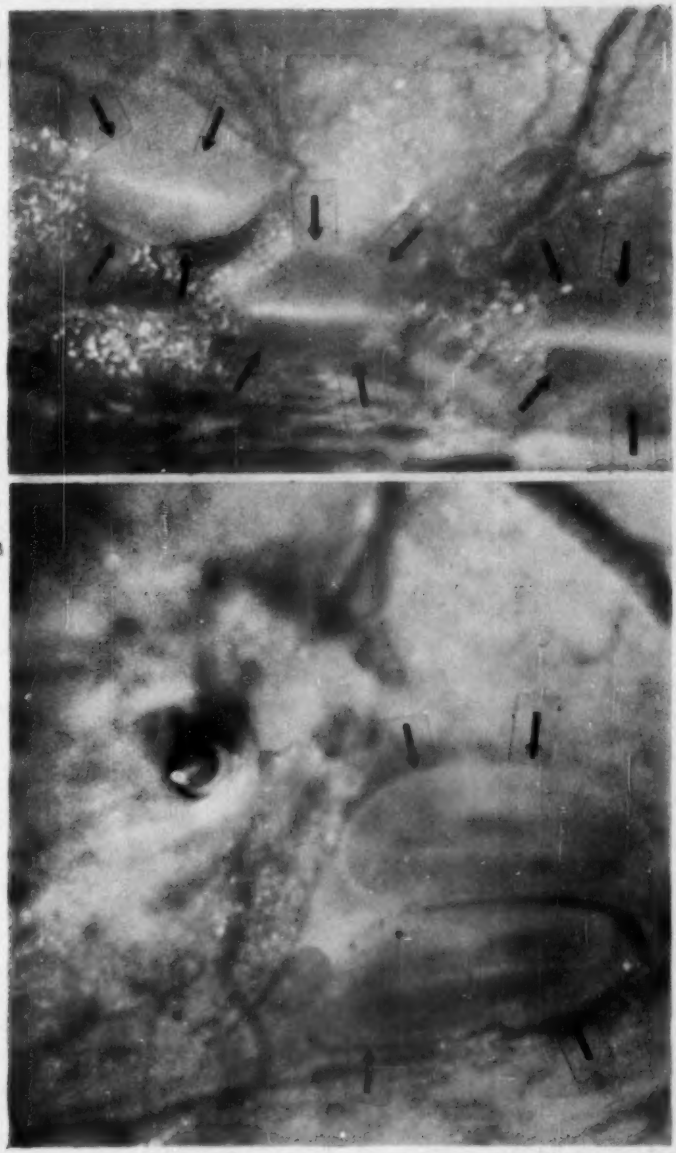
SCOTT, D. (1951). Response of Pacinian corpuscle to oscillatory stimulation. *Fed. Proc.* **10**, 123.

SKOGLUND, S. (1956). Anatomical and physiological studies of knee joint innervation in the cat. *Acta physiol. scand.* **36**, Suppl. 124.

## EXPLANATION OF PLATE

*A*. Photograph of three Pacinian corpuscles (↑) lying along tibia; note vessels running in tibial periosteum. Magnification × 30.

*B*. Photograph of under surface of skin in experiment of Text-fig. 5. *Note* needle point marking point c, and two adjacent Pacinian corpuscles. Magnification about × 50.




C. C. HUNT

(*Facing p.* 186)

*J. Physiol.* (1961), **155**, *pp.* 187–208
*With* 11 *text-figures*
*Printed in Great Britain*



# THE THERMOELASTIC EFFECT OF CHANGE OF TENSION IN ACTIVE MUSCLE

BY R. C. WOLEDGE

*From the Department of Physiology, University College London*

(*Received* 23 *September* 1960)

In spite of the large amount of work done on the subject, the interpretation of records of the heat produced in a muscle during an isometric twitch still remains somewhat doubtful. Immediately after the stimulus the rate of heat production is high, falling to a low value by the time the maximum tension is reached, but it then rises again as the tension falls (Hartree, 1931). Thus some 30–35% of the total heat is produced during relaxation, i.e. after the maximum tension has been passed. Does this heat production indicate that some chemical reaction occurs during relaxation? Some at least of this 'relaxation heat' must represent the mechanical potential energy lost by the muscle. Determination of the relaxation heat in a twitch is rather uncertain, because of the fact that the whole muscle does not relax simultaneously, so that the relaxation heat may be produced unevenly (Hill & Howarth, 1957). Nevertheless, Hartree (1931) obtained fairly precise figures for the total amount of relaxation heat. Consideration of these results shows that in an isometric twitch the mechanical potential energy degraded can account for only about half the heat produced during relaxation (Hill, 1953*b*). It was shown, however (Hill, 1949, 1953*c*), that if all the mechanical potential energy was removed from the muscle by an ergometer release which gradually reduced the tension to zero, no relaxation heat was produced. This strongly suggested that the remainder of the relaxation heat also had a physical cause, and was directly related to the fall of tension. In fact, Hill (1953*b*) showed that, in fully active muscle under a variety of conditions, a fall of tension is accompanied by immediate production of a proportional amount of heat. The mean relation found between fall of tension ($-\Delta P$) and the heat produced ($\Delta Q$) was $\Delta Q = -0{\cdot}018 . l_0 . \Delta P$, where $l_0$ is the 'standard' length of the muscle; the numerical constant in this equation is known as the 'thermoelastic heat: tension ratio'. During relaxation from an isometric twitch, that part of the heat produced which is not due to degradation of potential energy is close to the amount predicted from this formula. These results raise the question of whether a corresponding absorption of heat occurs during the initial development of tension. This would make the heat produced by

chemical reactions in the early part of the twitch appear smaller than it really is.

Hill interpreted the heat production during fall of tension as being of 'thermoelastic' origin. There are two main types of elastic bodies. In one type, often called normal, the elasticity is short-range; for example, a steel wire can only be stretched a short distance before it is permanently deformed. This type of material has a positive coefficient of thermal expansion; when it is put under tension heat is absorbed, and when the tension falls heat is given out. In the other type of elastic body, of which natural rubber is an example, there is long-range elasticity. These materials, when under tension, have a negative coefficient of thermal expansion; heat is absorbed when the tension falls, and given out when it rises. Resting muscle has rubber-like thermoelasticity (Hill, 1952) but the observation of heat production during fall of tension suggests that active muscle has 'normal' thermoelasticity. This conclusion is supported by studies of the thermoelastic properties of muscles in iodoacetate rigor (Aubert, 1956), in which it was shown that heat is produced during fall of tension and absorbed during rise of tension, though the thermoelastic heat: tension ratio was much smaller than in active muscles.

If the heat produced by fall of tension in active muscle is indeed thermoelastic, there must be a corresponding absorption of heat when the tension rises. The studies of Hill & Howarth (1959) provide an indirect indication that such an absorption of heat occurs. In their experiments active muscles were stretched, and the tension rose to very high values. The results were impossible to explain in any simple way unless it was assumed that rise of tension causes absorption of heat. Therefore the following study was undertaken of heat production in active muscle during rising tension, to see whether direct evidence of negative thermoelastic heat could be found. The natural rise of tension at the start of a contraction is unsuitable for this purpose, because the activation heat is produced at its greatest rate at the start of a contraction, and it is difficult to see what situation could be used as a 'control' in which the tension does not change but the activation heat is the same. Three types of experiment are reported in this paper: (1) a small stretch is applied to a muscle which is shortening isotonically, in order to make the tension rise rapidly; (2) a stretch is applied immediately after a quick release, bringing the muscle back to its original length; (3) tension is allowed to redevelop spontaneously after a small quick release.

Because an active muscle is continuously producing heat, the thermoelastic effect always has to be observed against a moving base line. This complication is reduced if the tension change is made rapid. This can be done, for a fall of tension, without causing any of the mechanical potential

energy lost by the muscle to appear as heat (Hill, 1953*b*). The main difficulty in the present investigation was that, whenever the tension in a contracting muscle was made to rise rapidly by applied stretch, as in experiments of types (1) and (2), extra heat appeared in the muscle, presumably derived from the work done in the stretch. It was well known (Abbott, Aubert & Hill, 1951; Hill & Howarth, 1959) that if the tension is artificially increased above $P_0$ (the maximum isometric tension in a tetanus), such 'irreversible' changes occur; but it was found that, even if the tension never rose above $P_0$, an applied stretch produced a similar effect, resulting in heat production which obscured, or tended to obscure, the expected absorption of heat. Because of this trouble, experiments of types (1) and (2) did not give any unequivocal evidence of thermoelastic heat. In the end, therefore, attention was concentrated on the spontaneous redevelopment of tension after a quick release. Since this necessarily involves shortening of the contractile component, the extra heat produced by this shortening tends to mask the thermoelastic effect. Hill has published an experiment of this type (1953*b*, Fig. 6) in which the release was made after the end of a tetanus; and so the amount of shortening heat was uncertain, and no allowance was made for it. In the present experiments the release and redevelopment of tension have been observed during a continuing tetanus, so that an allowance for the heat of shortening could be made with reasonable accuracy.

## METHODS

Sartorius muscles from English toads (*Bufo bufo*) have been used for all the experiments. The animals were kept in a cold store at 1° C, and were in good condition. For some experiments the nerves to the muscles were also dissected out and used for stimulation; this was particularly to avoid leak of the stimulating current, which, with direct stimulation, disturbed the records of heat production during a continuing tetanus. A pair of muscles was mounted on a thin thermopile. Two thermopiles have been used. With one of these (D 2, 1274 $\mu$V/° C), used only for the experiments in which a muscle was stretched during an isotonic contraction, stimuli were applied to the muscle through electrodes at each end of the thermopile. The equivalent half-thickness ($b$) of this thermopile, together with the epimysium, is estimated to be 22·4 $\mu$. The other thermopile (D 3, 3820 $\mu$V/° C) was used for all the other experiments. The advantage of its high sensitivity outweighed the disadvantage that it is thicker than D 2, the value of $b$, including 7 $\mu$ allowance for the epimysium, being 42 $\mu$. This thermopile (Fig. 1) is provided with electrodes for direct stimulation of the muscle and also with electrodes for nerve stimulation. These consist of two pairs of platinum wires, one pair for each nerve, mounted to one side of the thermopile and parallel to the muscle, so that small movements of the muscle can occur without damaging the nerves, or pulling the nerves or muscles out of position. The output of the thermopile was recorded by a sensitive and rapid galvanometer which, by means of photo-electric amplification, operated one beam of a double-beam oscilloscope; the other beam was used to record tension or length changes. The sweep of the oscilloscope could be started at any moment in relation to the stimulus or release.

The mechanical arrangements for the experiments in which a muscle was stretched during

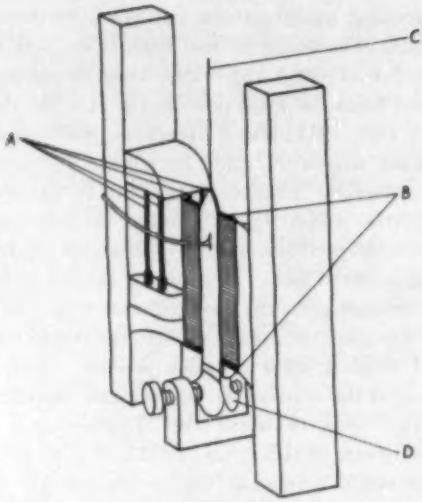



Fig. 1. Diagram of thermopile D3 with muscles and nerves in position. *A*, nerve-stimulating electrodes. *B*, electrodes for direct stimulation. *C*, wire connecting the muscles to the ergometer. *D*, pelvic bone held in clamp. For drawings showing details of thermopile construction see Hill (1938).

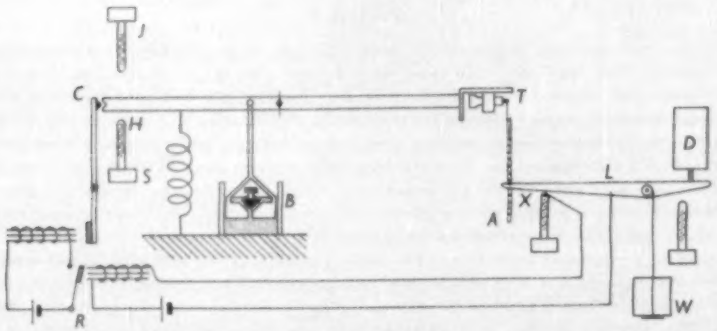



Fig. 2. Diagram of arrangement of apparatus for the experiments on stretching a muscle during an isotonic contraction. The muscles are connected to the chain at *A*, and contract against the isotonic lever (*L*), lifting the load *W*. When the isotonic lever makes contact with stop *X* the relay (*R*) causes the release of catch *C*, and the ergometer arm is then pulled down to stop *H* by the spring (*S*). The speed of the ergometer arm is varied by adjusting the needle valve in the dashpot (*B*). Tension during and after the stretch is recorded by the R.C.A. transducer (*T*). The movements of the isotonic lever are recorded by the displacement transducer (*D*). Diagram partly after Jewell (1960).

an isotonic contraction are shown in Fig. 2. The muscle was connected at *A* to the isotonic lever (*L*), and shortened against the load (*W*), until the lever made electrical contact with stop *X*, which, by way of the relay (*R*), released the catch (*C*). The released ergometer arm then moved down to stop *H*, first stretching the muscle, and then keeping it isometric. The tension was recorded during and after the stretch by the transducer mounted on the arm of the ergometer. The chain connecting the transducer to the isotonic lever was just long enough to allow the lever to make contact with stop *X*. For the contractions which were isotonic throughout, stop *X* was screwed down, and the ergometer arm was held against stop *J*, so that the muscle continued to shorten against the isotonic lever, the movements of which were recorded by the transducer *D* (J. Langham Thompson, type 542/5).

The Levin Wyman ergometer was used also for the experiments in which a muscle was released. The spring (*S*) was arranged to pull the ergometer arm upwards towards stop *J*, thereby releasing the muscle. The relay (*R*) was replaced by a key, which could be opened at any moment after stimulation began. The transducer on the ergometer arm was connected direct to the muscle by stainless steel wire (33 s.w.g.) weighing about 0·2 g. The extensibility of this wire between 80 and 20 g was 0·04 mm; the extensibility of the transducer and its connexions between these limits was 0·08 mm.

The experiments on rapid release followed immediately by rapid stretch were made with a device constructed by modifying a Carpenter relay (type 3E1). The tip of the armature was extended about 20 mm by a light alloy arm, to the end of which the muscle was attached. The contacts were screwed back until the armature rested on a thin layer of aluminium foil inserted between the armature and the magnet. The movement of the end of the alloy arm was then 0·57 mm when the armature moved from one side to the other. The coils were connected in two separate groups, of which one moved the armature over to release the muscle, and the other returned it to its starting position. Current was supplied to the coils through condensers. The time constant of the circuit for the initial downward movement of the armature was made smaller than that of the circuit for the return movement, so that if current was supplied to both circuits together the armature moved down and returned without the need for any additional switching. The device was capable of releasing the muscle and returning it to its initial length in less than 10 msec. The moving arm was too small to carry an R.C.A. transducer, so tension could not be recorded at the same time as heat production. Tension records could be made separately, however, by connecting the pelvic end of the muscle to an R.C.A. transducer, the electrical connexions of which were 'potted' in 'Araldite' so that it could be used in Ringer's solution.

In a few experiments the records of heat production were analysed in the usual way, to allow for the time lag in thermopile and galvanometer. For the most part, however, this procedure would have been difficult and somewhat inaccurate, because of the speed of some of the changes and because the thermopile D3 is rather slow. Fortunately it was possible to reverse the procedure and to calculate, from the observed tension changes, what the thermoelastic heat deflexion would be, for comparison with the observed heat deflexion. The assumption made in the calculation was that the rate of tension change, positive or negative, was accompanied by a proportional rate of heat production, negative or positive respectively. If the deflexion so calculated agreed with that observed experimentally, the assumption was confirmed just as effectively as it would be if the result of analysing the heat records agreed with the observed changes of tension. The advantage of using a 'synthesis' is that it is much more accurate than an 'analysis' and can be carried out in any intervals desired, however short. An analysis would have been difficult and inaccurate, even with intervals as long as 20 msec, while in the synthesis 4 msec intervals were used. (As will be seen in the figures that follow, 20 msec is rather a long time in the rapid changes involved.) The method of synthesis has seldom been used in the past because the 'expected' result could not be stated beforehand in a quantitative way, as is possible in these experiments.

The procedure of synthesis, or numerical integration, is carried out with the usual

numerical data characteristic of thermopile and galvanometer. With 4 msec intervals a table is constructed giving the deflexion of the galvanometer at 2, 6, 10, ... msec after the start of a constant current. For the thermopile a similar table is constructed for the current due to an instantaneous rise of temperature in the muscle. These two tables are then combined to give the deflexion of the galvanometer at 2, 6, 10, ... msec after an impulsive change of temperature. From this table it is a simple matter to calculate, with the aid of an adding machine, the deflexion of the galvanometer for a change of temperature of any given form. The change of temperature is considered as a series of instantaneous changes of suitable size, occurring at 2, 6, 10, ... msec after a convenient zero. The result of the calculation is then the response of the galvanometer at 4, 8, 12, ... msec after this zero.

In the experiments in which a muscle redeveloped tension spontaneously after a release, a rather substantial correction is required for the heat of shortening. This was calculated from the observed redevelopment of tension in the following way. The total compliance of the muscles and the connexions to the transducer is found from the drop of tension during the release. The amount of active shortening during a release of about 0·5 mm at 70 or 80 mm/sec is small, and may be estimated without significant error as 0·02 mm. In each experiment the compliance was found from a release of about this speed. After allowing for this shortening, the total compliance over the required range is known. Allowance is made for the non-linearity of this compliance by calculating the load–extension curve for the muscle from the generalized curve given by Jewell & Wilkie (1958), and adding to it a sufficient linear compliance, representing the connexions and the transducer, to bring the total to the observed value. By means of this load–extension curve, the time course of the shortening is obtained from the recorded rise of tension. Next, the galvanometer deflexion which would result from a heat production of this form is calculated by the method just described. The size of this deflexion is found by assuming a value of the constant $a$ (the amount of heat produced by unit shortening). It is known that $a$ is between $P_o/4$ and $P_o/5$ (Hill, 1938; Abbott, 1953). A convenient value between these limits is assumed. The deflexions calculated in this way were subtracted from those observed at the same times after release, to give a corrected curve. It was this curve that was compared with the thermoelastic heat deflexion calculated from the changes in tension.

## RESULTS

### *Heat production during spontaneous redevelopment of tension after a quick release*

Figure 3, curve *a*, shows the mean of three records of the heat production in tetanic contractions of a pair of toad sartorii at 0° C. The sweep of the oscilloscope started 0·74 sec after the beginning of the tetanus, when the heat record was already 35·7 mm above its base line. At 0·80 sec the muscle was released 0·4 mm at a rate of 35 mm/sec, and the tension fell as shown in curve *c*. The mean of two heat records in which the muscle was isometric throughout is also shown (curve *b*). During the release the tension fell from 91 to 34 g, and then rose again during the next 0·38 sec to almost its original level. At the same time the heat record diverged from the isometric record, and then gradually returned towards it. If the ordinates of the isometric heat record are subtracted from those of the record for a release, curve 1 in Fig. 4 is obtained. (Whether this subtraction is justified will be discussed later.)

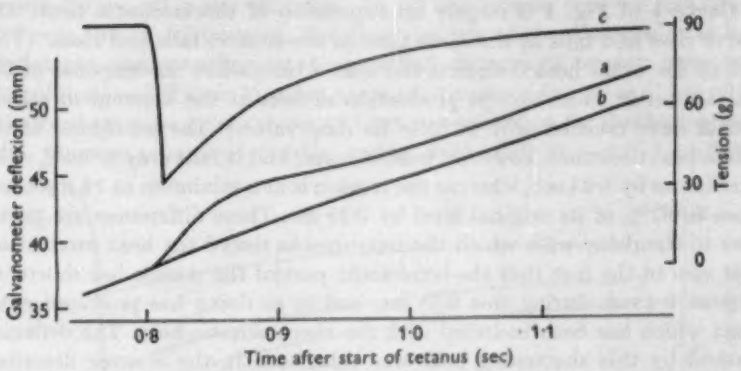



**Fig. 3.** Simultaneous records of heat production (*a* and *b*) and tension (*c*) in tetanic contractions of toad sartorii at 0° C. Muscles weighed 91 mg, $l_0$ = 25·5 mm. Nerve stimulation was used; the tetani were of one shock every 60 msec, and of 1·1 sec duration. Ordinate, tension and galvanometer deflexion; sensitivity of heat recording, heat equivalent to 0·28 g.cm would produce 1 mm steady deflexion, neglecting heat loss. (In the following legends the sensitivities are expressed in the same way, but written in the short form of '1 mm ≡ 0·28 g.cm'.) (*a*) and (*c*): muscles released 0·5 mm; mean of records 1, 3, 5 in series. (*b*): muscle isometric; mean of records 2 and 4.

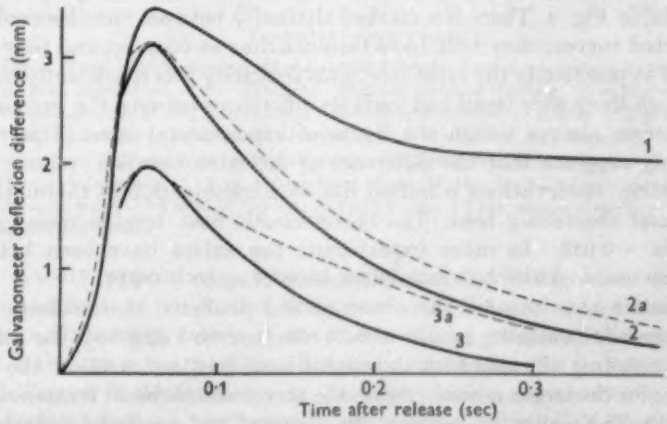



**Fig. 4.** Extra heat deflexion following release. Same muscles as Fig. 3. Ordinate: galvanometer deflexion difference (1 mm ≡ 0·28 g.cm). (1) 0·5 mm release, same observations as Fig. 3; difference between ordinates of the 'release' record and those of the 'isometric' record. (2) line (1) minus calculated shortening heat deflexion. (3) extra heat deflexion after 0·26 mm release minus calculated shortening heat deflexion. Interrupted lines (2*a* and 3*a*) are thermoelastic heat deflexions calculated from the appropriate tension records, taking the thermoelastic heat: tension ratio as −0·013.

Curve 1 of Fig. 4 is largely an expression of thermoelastic heat. The curve rises and falls at the same time as the tension falls and rises. (This fall of the extra heat deflexion starts at a time when the response of the galvanometer to an abrupt production of heat at the moment of release would have reached only 80% of its final value.) The maximum of the extra heat deflexion, however, is at 60 msec, and it falls only to 60% of its maximum by 0·38 sec, whereas the tension is at a minimum at 12 msec and rises to 97% of its original level by 0·38 sec. These differences are partly due to the delay with which the instruments record the heat production, but also to the fact that the contractile part of the muscle has shortened almost 0·4 mm during this 0·38 sec, and in so doing has produced extra heat which has been included with the thermoelastic heat. The deflexion caused by this shortening heat was calculated in the manner described above, taking $a$ as 18 g wt., and was subtracted from curve 1 (the extra heat deflexion observed to follow the release). The result is shown as curve 2 in Fig. 4.

In order to compare this heat deflexion with the fall in tension it is assumed that every element of tension change causes a proportional heat production, and a synthesis is made as described above. The resulting theoretical thermoelastic heat deflexion is adjusted in size to make its maximum the same as that of curve 2. The result is shown by the broken line, 2$a$, in Fig. 4. There is a marked similarity between the observed and predicted curves: they both have their maxima at 60 msec, and they then fall off at practically the same rate. This similarity was consistently found; although there were small and variable differences between the two curves, these were always within the limits of experimental error. This result strongly suggests that the difference of deflexion between 'release' and 'isometric' observations is indeed due to a combination of thermoelastic heat and shortening heat. The thermoelastic heat:tension ratio in this case is $-0{\cdot}013$. In other experiments the values have been between $-0{\cdot}009$ and $-0{\cdot}018$, but have often been close to $-0{\cdot}014$.

Figure 4 also includes the observed and predicted thermoelastic heat deflexions for a smaller release of 0·26 mm (curves 3 and 3$a$). The calculation, including allowance for shortening heat, has been made in the same way as for the larger release. Again the thermoelastic heat: tension ratio is $-0{\cdot}013$. The similarity between the observed and predicted deflexions is not so close as for the large release but the experimental errors tend to be proportionally larger for the smaller amount of heat.

Figure 5 shows the result of an experiment in which twitches were used. It is similar to the experiments reported by Hill (1953$c$). The extra heat deflexion which followed releases of three different distances is shown, together with the corresponding tension records. The releases were all

made 0·4 sec after the stimulus. In (*a*) the release was 0·3 mm, in (*b*) 0·6 mm and in (*c*) 0·9 mm. The time courses of the thermoelastic heat deflexions corresponding to the observed changes of tension have been calculated, and if their maxima are made the same in size as those of the observed extra heat deflexions, without any correction for shortening heat, the thermoelastic heat:tension ratios work out at −0·012, −0·013,

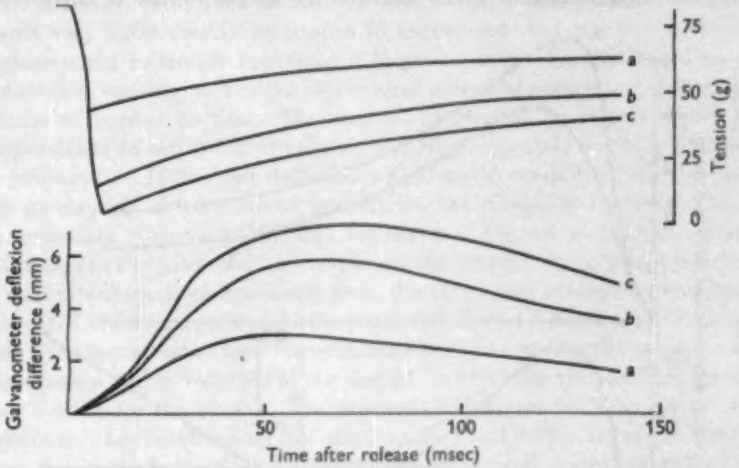



Fig. 5. Extra heat deflexion and tension after releases of various distances. Twitches of toad sartorii at 0° C. distances. Muscles weighed 140 mg; $l_0$ = 27·5 mm. Ordinate: tension and galvanometer deflexion difference (1 mm ≡ 0·23 g.cm). Each tension curve is the mean of three 'release' records (1, 3, 5 in series). Each extra heat deflexion curve is obtained by subtracting the mean ordinates of two 'isometric' records (2, 4 in series) from those of the three 'release' records. Releases were (*a*) 0·3 mm, (*b*) 0·6 mm and (*c*) 0·9 mm. All the release started 0·4 sec after the stimulus.

and −0·013 respectively. The value of the shortening heat constant, *a*, is uncertain so long after the stimulus as 0·4 sec, so detailed comparison of the time course of the observed and predicted thermoelastic heat deflexions is not possible. But it is evident that the curves in Fig. 5 are falling off in the same way as the corresponding curves obtained from observations during a continuing tetanus (Fig. 4, curve 1). If *a* were taken as $P_0/4$, the heat:tension ratios would be −0·011, −0·012 and −0·011, after making allowance for the shortening heat. The true values of the ratios will lie somewhere between these two estimates.

Figure 6*a* (curves 1 and 2) shows the extra heat deflexions which followed releases made at two different speeds. Each curve was obtained by subtracting the mean ordinates of six 'isometric' records from those

of six records of the release. The releases were made during a tetanus, and the extra heat deflexion curves have been corrected for shortening heat as described above. It is seen that the release at 80 mm/sec (1) produced 30% more extra heat deflexion than the release at 25 mm/sec (2), although the fall in tension was only 10% greater. The broken lines in the figure show the thermoelastic heat deflexions predicted from the tension records. The extra heat deflexion which followed the slower release is fairly close

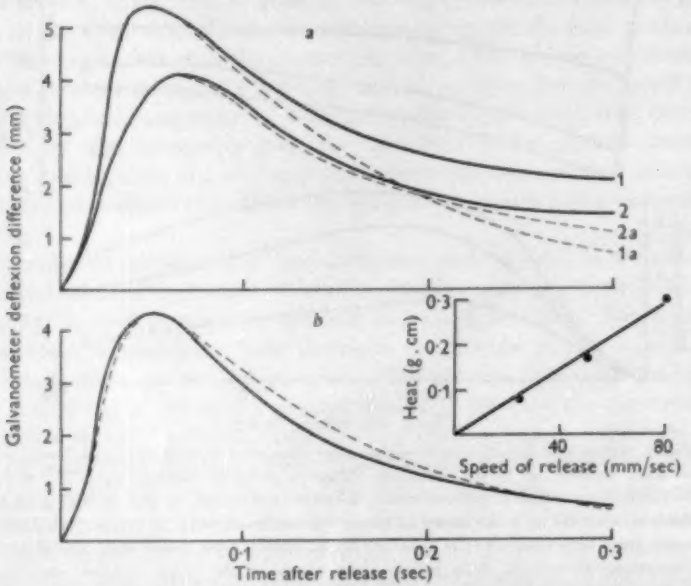



Fig. 6. Extra heat deflexion following releases at different speeds, corrected for shortening heat. Toad sartorii at 0° C. Muscles weighed 68 mg; $l_0$ = 24 mm. Nerve stimulation; tetani of 1·1 sec, one shock every 60 msec. Releases 0·8 sec after start of tetanus, all 0·4 mm. Ordinate: heat deflexion difference (1 mm ≡ 0·18 g.cm).

(*a*) (1) release at 80 mm/sec. (2) release at 25 mm/sec. Each line is obtained by subtracting the mean ordinates of six 'isometric' records (2, 4, 6, 7, 9, 11 in series) from those of six 'release' records (1, 3, 5, 8, 10, 12), and subtracting the calculated shortening heat deflexion. Interrupted lines (1*a*) and (2*a*) are thermoelastic heat deflexions calculated from the appropriate tension records and adjusted in size to make the maxima the same as those of curves (1) and (2).

(*b*) Full line is (1) of (*a*) minus the deflexion calculated for 'irreversible' production of 0·3 g.cm of heat during the release. Interrupted line is the thermoelastic heat deflexion calculated from the tension record and adjusted in size to make its maximum the same as that of the full curve.

Inset: ordinate 'irreversible' heat production giving deflexion which, when subtracted from the corrected extra heat deflexions, gives the best fit with the predicted thermoelastic heat deflexion (see text); abscissa, speed of release.

to the predicted time course, but the deflexion which was produced by the faster release does not fall off so rapidly as the predicted thermoelastic heat deflexion, being 1·5 mm larger by 0·3 sec after the release. In this experiment a set of records was also made at a speed of release of 50 mm/sec. This gave an extra heat deflexion intermediate in size between the other two, which was 0·7 mm larger than the predicted thermoelastic heat deflexion at 0·3 sec.

It is known (Hill 1953*a*) that the series elastic component of muscle shows very little viscous resistance to movement, but the above discrepancies could be simply explained if there was some viscous resistance in the system, causing part of the mechanical potential energy lost during the release to appear as heat. The amount of heat so produced would be proportional to the speed of release, and its production would, of course, be irreversible. If the heat deflexion which would result from the irreversible production of 0·3 g.cm of heat during the release is subtracted from the ordinates of curve 1 (Fig. 6*a*), the curve of Fig. 6*b* is obtained, which fits closely to the predicted time course for the thermoelastic heat deflexion. If similar subtractions are made from the other two observed extra heat curves to obtain the best fit with the predicted thermoelastic heat deflexions, then the thermoelastic heat:tension ratio becomes nearly the same for all the releases, being $-0{\cdot}016$ for the fastest, $-0{\cdot}015$ for the medium speed, and $-0{\cdot}014$ for the slowest. The amounts of irreversible heat giving the deflexions which were subtracted are 0·30, 0·16 and 0·08 g.cm respectively. Thus the amount of irreversible heat produced is nearly proportional to the speed of release, as is shown in the inset to Fig. 6*b*. The line in this graph represents a viscous resistance of 600 dyn/(cm/sec). In two other experiments largely similar results were obtained, but not with sufficient accuracy to justify calculation for the viscous resistance.

Hill (1953*a*) found that only about 4% more work was obtained by releasing a muscle at 140 mm/sec than by a release at 40 mm/sec. This is equivalent to a viscous resistance of less than 100 dyn/(cm/sec). These experiments were performed with the muscle in Ringer's solution on a multi-electrode assembly. Under these conditions the friction between the muscle and the multi-electrode assembly is negligible, but in the present experiments the muscle was in oxygen and separated from the surface of the thermopile by only a thin layer of fluid. Any 'friction' between the muscle and the thermopile will be due to the shearing of this layer of fluid, and so will be a viscous resistance to the movement of the muscle.

To obtain a direct measurement of the total viscous resistance to movements of the muscles on the thermopile in air, the experiment shown in Fig. 7 was performed. During a tetanus the muscles pulled on an isotonic

lever, loaded with 11 g wt., and held in position by an electromagnetic catch keeping the muscle isometric. At 0·6 sec after the start of the tetanus the catch was abruptly withdrawn, and the movements of the

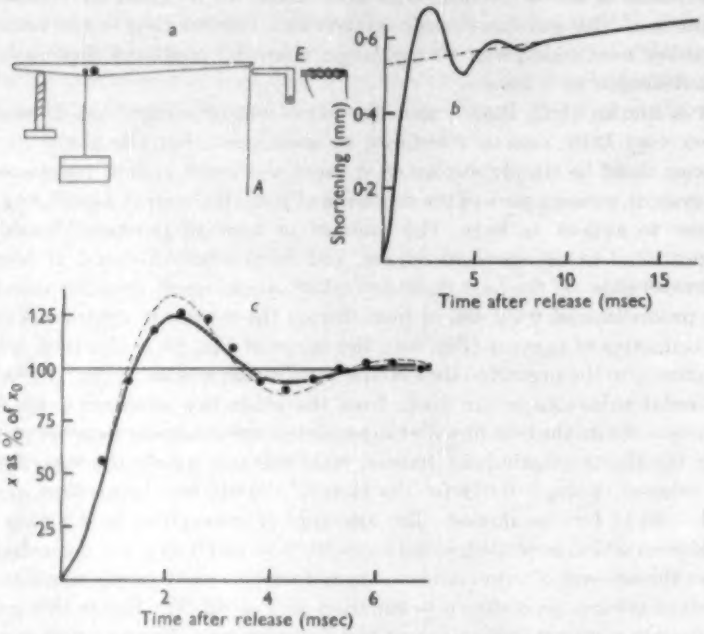



Fig. 7. (*a*) Diagram of arrangement of apparatus for isotonic quick-release experiments. The muscles pull on the isotonic lever by the wire (*A*), but the lever is held in position by the electromagnetic stop (*E*), which is released during a tetanus. The movements of the isotonic lever are recorded by a photoelectric system.

(*b*) Experimental record of the shortening following the release of the isotonic lever 0·6 sec after the start of a 0·8 sec tetanus. The load on the muscles after release was 11 g. Toad sartorii at 0° C; weight: 60 mg; $l_0$ = 26 mm. The isometric tension was about 60 g. The interrupted line is an extrapolation of the linear part of the record and shows the active shortening assumed to have occurred.

(*c*) Lines calculated from $x = x_0[1-\{e^{-bt}/\sqrt{(k^2-b^2)}\}.\cos(\sqrt{(k^2-b^2)}.t-\theta)]$; where $x$ is the distance moved by time $t$ (sec) and $x_0$ is the final displacement; $k = \sqrt{(1/MC)}$; $b = R/2M$; $\sin\theta = b/k$; $M$ (mass) = 0·4 g; $C$ (compliance) = $10^{-6}$ cm/dyne; $R$ (viscous resistance) = 500 dyn/(cm/sec) for the full line and 400 dyn/(cm/sec) for the interrupted line. The points ● are the observed oscillations after correction for active shortening, expressed as % of final value.

lever were then recorded. The record is shown in Fig. 7*b*. There are two phases of shortening; first the rapid shortening of the series elastic component, then a slower, active shortening of the contractile component. The transition between the two is obscured by a damped oscillation (Jewell &

Wilkie, 1958; Jewell, 1959). This oscillation is due to sudden release of a system containing compliance, largely situated in the muscle, in series with a mass, the lever and connecting wire. The damping of the oscillations is due to the viscous resistance to movements of the muscles (the resistance to movement of the lever is known to be only about 50 dyn/(cm/sec)).

The equivalent mass of the lever, referred to the point of attachment of the muscle, has been determined by Jewell (1959), and is 0·2 g. The connecting wire weighs 0·2 g. The compliance of the system is known from the extent of the rapid phase of the movement after release, and is taken as $10^{-6}$ cm/dyne. The oscillations which would occur after release of a mass of 0·4 g in series with a compliance of $10^{-6}$ cm/dyne were calculated for two values of the viscous resistance, 400 and 500 dyn/(cm/sec), and the result is shown by the two lines in Fig. 7*c*. The oscillations observed were corrected for active shortening of the muscle by assuming that this starts immediately after the release at the rate observed when the oscillations have died out (Jewell & Wilkie, 1958). As is shown in Fig. 7*c*, the observed oscillations are then very similar to those calculated, and the value of the viscous resistance is about 500 dyn/(cm/sec).

This experiment shows that the value of the viscous resistance suggested to explain the results of the experiment of Fig. 6 is quite likely, and so the explanation is probably correct. It is not clear whether the difference between this result and that of Hill (1953*a*) is entirely due to the fact that in the present experiments the muscles were mounted on a thermopile in air. J. Délèze and D. R. Wilkie (personal communication) have made similar experiments with single frog sartorius muscles on a multi-electrode assembly in Ringer's solution and have found values for the viscous resistance of about 200 dyn/(cm/sec).

*Effects of a small stretch during an isotonic contraction*

The obvious method of observing the thermoelastic heat absorption due to rise of tension would be to give the contracting muscle a stretch, which would cause the tension to rise rapidly. But if the tension rises above $P_0$ (the maximum tension which the muscle can exert in a tetanus) a large amount of heat will be produced (Hill & Howarth, 1959), and this will obscure the expected absorption of heat. On the simple model of active muscle as a contractile component in series with an elastic component, the heat produced when the tension rises above $P_0$ is due to lengthening of the contractile component. Experiments were therefore made in which a muscle, shortening isotonically with a load of about $P_0/4$, was given a small stretch, about 0·5 mm, sufficient to raise the tension almost to $P_0$, but not above. The length change in the muscle should then have taken place only in the series elastic component.

Two experiments of this type are shown in Fig. 8. In each case the recorded heat deflexion is shown during an isotonic contraction in which the shortening was interrupted by a stretch, and the muscle was then kept isometric. In the experiment shown in Fig. 8*a* the stretch was completed within 20 msec; extra heat appeared in the muscle. This extra heat production was seen whenever a muscle was stretched at more than about 10 mm/sec. This heat is presumably derived from the work done in stretching the muscle. Since, as described above, the series elastic part of the muscle has a viscous resistance to movement of about 500 dyn/(cm/sec),

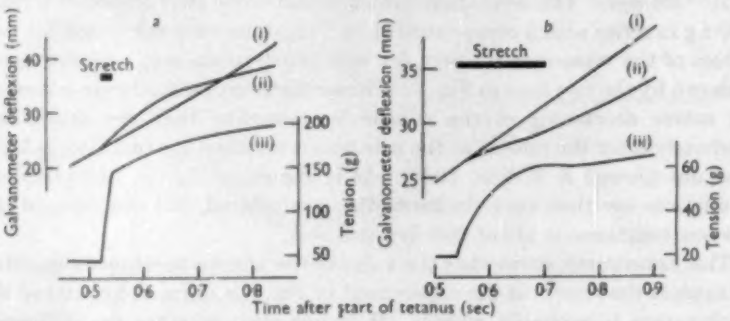



Fig. 8. Records of heat production, (i) and (ii), and tension, (iii), during tetanic contractions of toad sartorii at 0° C. Tetani: one shock every 60 msec; 1 sec duration. (i) isotonic contraction; (ii) and (iii) isotonic contraction interrupted by stretch.
(*a*) Isotonic load 45 g wt; stretched 0·8 mm in 20 msec; muscles weighed 361 mg; $l_0$ = 37 mm. Sensitivity of heat recording: 1 mm ≡ 1·4 g.cm.
(*b*) Isotonic load 18 g wt; stretched 0·5 mm in 160 msec; muscles weighed 108 mg; $l_0$ = 25·5 mm. Sensitivity: 1 mm ≡ 0·50 g.cm.

the heat produced by this resistance during the stretch shown in Fig. 8*a* would be about 0·16 g.cm, whereas about 3 g.cm of extra heat was actually produced. It seems, therefore, that the contractile component, or part of it, is lengthening during the rapid rise of tension, although the tension is always less than $P_0$.

Figure 8*b* shows an experiment in which a muscle, shortening isotonically at 7·8 mm/sec, was stretched 0·5 mm at 3·5 mm/sec. The heat production of the muscle when stretched was always less than in the isotonic control. The ordinates of the heat record during and after the stretch were subtracted from those of the isotonic control, and the resulting deflexion difference was analysed, at 20 msec intervals. The result is shown in Fig. 9. Most of this difference of heat production is due to the shortening heat produced in the isotonic contraction. However, the shortening during this 0·3 sec occurred at a constant rate, whereas the curve showing the difference of heat production rises most rapidly during the stretch itself.

That is, the heat production in the stretched muscle diverges from the isotonic control more rapidly than would be expected from the difference in shortening alone. This is due to the absorption of some heat during the stretch.

If the shortening in the stretched muscle stopped abruptly at the moment when the stretch began, the amount of heat absorbed by the stretched muscle could be found by extrapolating the linear portion of the curve in Fig. 9. The intercept on the $y$ axis is found to be 0·44 g.cm. If this were thermoelastic heat absorption, the heat:tension ratio would be

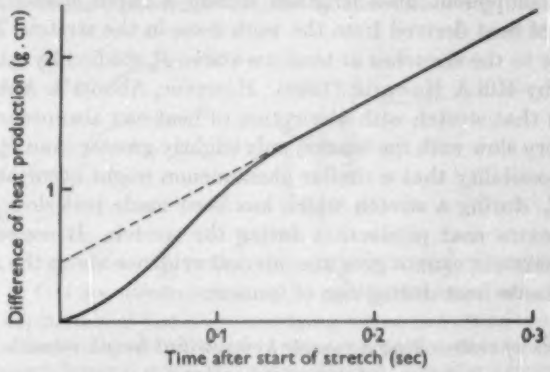



Fig. 9. Difference of heat production during and after a stretch. Same observations as Fig. 8*b*. The ordinates of the record of heat deflexion during and after the stretch (8*b*, ii) were subtracted from those of the 'isotonic' record (8*b*, i) and the resulting difference of deflexion was analysed using 20 msec intervals. The result is shown by the solid line. The interrupted line is an extrapolation of the linear part of the difference of heat production. The duration of the stretch was 160 msec.

−0·004. If the shortening in the stretched muscle continued to some extent after the beginning of the stretch, as would be expected, some shortening heat would be produced during the stretch and would obscure part of the thermoelastic heat absorption. The force–velocity relation of the muscles used in this experiment was found by observing the velocity of shortening under various isotonic loads. The shortening which would have occurred during the stretch was then calculated on the assumption that the same relatior between force and velocity applies under these conditions. It was found that the muscle would have shortened 0·08 mm, which would produce about 0·17 g.cm of heat. The addition of this amount to the thermoelastic heat would make the heat:tension ratio −0·006.

This experiment shows that heat is absorbed during a stretch, but the amount of heat is difficult to measure accurately, partly because the amount of shortening during the stretch is not known. This uncertainty is

made worse by the need to use only slow stretches to avoid the extra heat production associated with rapid stretch. Another difficulty is that heat production from this cause may not have been altogether avoided in the slower stretch, and might have obscured part of the thermoelastic heat absorption. This may explain why the thermoelastic heat:tension ratio found in the above experiment was so small.

Yet another difficulty in interpreting this experiment is the possibility that heat might be absorbed by a small lengthening of the contractile component during the stretch. As described above, it seems likely that the contractile component does lengthen during a rapid stretch, with the production of heat derived from the work done in the stretch. This seems to be similar to the stretches at tensions above $P_0$ studied by Abbott *et al.* (1951) and by Hill & Howarth (1959). However, Abbott & Aubert (1951) have shown that stretch with absorption of heat can also occur when the stretch is very slow with the tension only slightly greater than $P_0$. It seems at least a possibility that a similar phenomenon might occur at a tension less than $P_0$, during a stretch which has been made just slow enough to avoid any extra heat production during the stretch. It seems that this type of experiment cannot give unequivocal evidence about the absorption of thermoelastic heat during rise of tension.

*Effects of restretching a muscle to its initial length immediately after a rapid release*

It had been suggested by Dr D. R. Wilkie (Hill, 1953*b*, p. 171) that a muscle might be stretched with fewer complications if the stretch were applied immediately after a release. If the stretch were of the same distance as the preceding release, the tension should return exactly to its former level, and the net thermoelastic heat would be zero. For this purpose the apparatus described under 'Methods' was made. It is capable of releasing a muscle and restretching it to its initial length within 10 msec. Figure 10*A* shows the heat deflexion during a release-and-stretch of 0·56 mm made in 9 msec, compared with the record for a release only. It is clear that the release-and-stretch produced more heat than the release alone. (Twitches were used in the experiment shown in this figure; similar results have been obtained during a continuing tetanus.) It was possible that the muscle had shortened a small amount during the few milliseconds after the release, so that the stretch would raise the tension above $P_0$, and so cause lengthening of the contractile component.

To investigate this possibility, a number of tension records were made during a release-and-stretch. This could not be done at the same time as the heat measurement, since the tension had to be recorded from the pelvic end of the muscle. A typical record is shown in Fig. 10*B*, for a release-

and-stretch of 0·56 mm. The tension at the end of the stretch had indeed returned exactly to $P_0$, but during the next 60 msec it fell to a minimum of 7 g less than $P_0$, then rose again. It is clear that the contractile component must have been lengthening during the fall of tension following the stretch, so that the mechanical potential energy lost must have appeared in the muscle as heat. This must be one cause of the heat production observed to follow the release-and-stretch. The mechanical potential energy lost in the case shown in Fig. 10*B* is about 0·5 g.cm. Another source of heat will be the viscous resistance of 500 dyn/(cm/sec) discussed above. Release and restretch each occur at about 20 cm/sec, so that about 1 g.cm of heat will

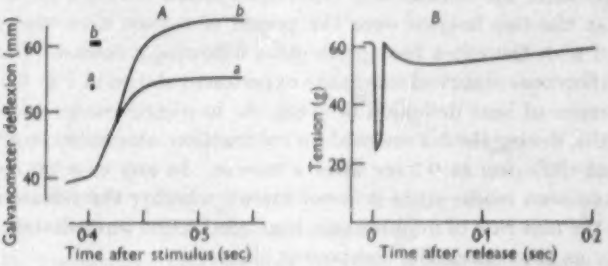



Fig. 10. (*A*) Records of heat production during release and release-and-stretch. Twitches of toad sartorii at 0° C. Muscles weighed 123 mg; $l_0$ = 26·5 mm. Ordinate: galvanometer deflexion (1 mm ≡ 0·19 g.cm). Abscissa: time after stimulus. *a*, release only of 0·56 mm. *b*, release-and-stretch of 0·56 mm. Bars show the duration of release and release-and-stretch.

(*B*) Tension record during and after a release and stretch of 0·56 mm. Tetanus of toad sartorii at 0° C. Muscles weighed 73 mg; $l_0$ = 25·5 mm. Release started 0·28 sec after the start of a 1·1 sec tetanus.

be produced. These figures are not directly comparable to the 3·5 g.cm of heat observed to follow release-and-stretch shown in Fig. 10*A*, since they were obtained in a different experiment. It is, however, clear that heat production from these and possibly other causes makes it impossible to tell whether or not there is any net thermoelastic heat production over the cycle of release-and-stretch.

## DISCUSSION

The most important part of the results described above is the observation that the extra heat deflexion following a quick release reaches its maximum value in about 60 msec, and then falls off. The only treatment of the results required to bring out this fact is the subtraction of the ordinates of the isometric heat record from those of the record for a release. The assumption made in this subtraction is that the processes which produce heat in the isometric muscle continue at the same rate if the

muscle is released. While the isometric tension remains constant the only heat production is the maintenance heat, so that the assumption seems likely to be correct. The rate of maintenance heat production during a tetanus does depend on the length of the muscle, being greatest at $l_0$ and falling off at both greater and smaller lengths (Aubert, 1956); but a change of length of only about 0·5 mm in the region of $l_0$ would be expected to produce hardly any change. However, a very small alteration in the rate of maintenance heat production might produce a noticeable error over a period as long as 0·3 sec. Therefore, as a further check the isometric contractions were made alternately at the lengths corresponding to those before and after the release. The differences found between the heat production at the two lengths over the period of 0·3 sec were always small compared with the extra heat production following a release. One of the largest differences observed was in the experiment shown in Fig. 6, in which the difference of heat deflexion between the isometric contractions at the two lengths, during the 0·3 sec used for subtraction, amounted to 5 % of the extra heat deflexion at 0·3 sec after a release. In any case no correction could have been made, since it is not known whether the released muscle takes up the new rate of maintenance heat production immediately, or only gradually as the contractile component shortens.

In the case of observations made during a twitch or after the end of a tetanus, the isometric tension tends to fall and, as it does so, extra heat is produced, partly thermoelastic and partly due to degradation of potential energy. This heat production does not occur after a release, so that comparisons of the isometric and release records are only justifiable during the limited time that the isometric tension remains almost constant. This is another reason why the most accurate results are obtained when the experiment is made in the course of a tetanus.

As described above, the detailed comparison of the observed and predicted thermoelastic heat deflexions depends on knowing the values of two constants, $a$, the heat produced by unit shortening, and $b$, the equivalent half-thickness of the thermopile. A value of $a$ has been assumed for each experiment somewhere between $P_0/4$ and $P_0/5$ (Hill, 1938). For example, in the experiment shown in Fig. 2 $a$ has been taken as 18 g wt., but it might really have been 23 g wt. Thus there is an uncertainty in the value of the observed thermoelastic heat deflexion which becomes progessively greater as shortening proceeds. At 60 msec after the release the uncertainty of curve 2 in Fig. 2 is only 0·1 mm (3%), but by 0·38 sec the uncertainty has become 0·5 mm. A possible error of this size means that an exact fit of the observed and predicted thermoelastic heat deflexions must not be expected, but it is not large enough to obscure their basic similarity.

To find out whether an error in the value (42 $\mu$) of the constant $b$, which

is the equivalent half-thickness of the thermopile plus an allowance for the thickness of the epimysium, would have upset the results, a typical thermoelastic-heat deflexion was recalculated for $b = 50\ \mu$. The result is shown in Fig. 11, which shows that this alteration in $b$ makes rather little difference. It seems that any likely error in the value of $b$ would have only a small effect on the results.

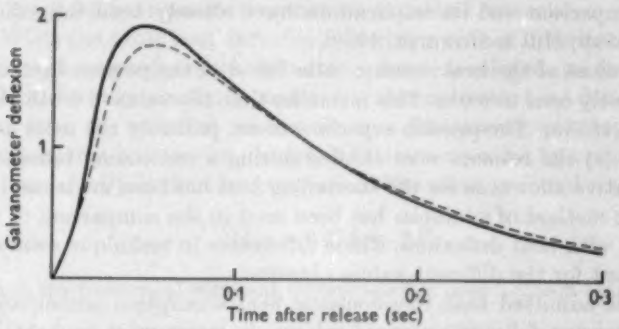



Fig. 11. Thermoelastic heat deflexions calculated from a typical record of the tension changes after a small quick release. Ordinate: galvanometer deflexion in arbitrary units. Solid line calculated for a value of the equivalent half thickness of the thermopile ($b$) of $42\ \mu$; interrupted line for $b = 50\ \mu$.

It is difficult to think of any explanation of the falling off of the extra heat deflexion following a release other than that it is due to the absorption of thermoelastic heat. It might be argued that the part of the muscle next to the thermopile was exerting more tension than the rest. The effect of this on the record would be that the thermoelastic heat deflexion would rise above its mean value and then fall back to this level. An example of this phenomenon is given by Hill (1953*b*, Fig. 7). With the direct stimuli applied through only two electrodes, on one surface of the muscle, it was possible that some of the fibres on the other surface might not be stimulated; but with nerve stimulation there is no reason why this should happen. In fact direct stimuli probably do stimulate the whole muscle, since Hartree (1929) showed that the heat deflexions produced by direct and indirect stimulation of a muscle were identical. The muscles used probably did have individual inequalities and these may well explain some of the differences found between the observed and predicted thermoelastic heat deflexions, but they could not have been responsible for the typical falling off of the extra heat deflexion. The chief reason for believing that decrease of the extra deflexion is due to the absorption of thermoelastic heat is the consistency with which the expected time course is found. It is

scarcely conceivable that a quite different cause could give a deflexion always so close to that predicted from the tension record.

The present experiments merely confirm the existence of the thermoelastic heat, they do not add anything to an understanding of the mechanisms producing it. The best explanation still seems to be the comparison with thermosetting plastics, wood, and ebonite, all of which have thermoelastic heat:tension ratios similar to that found in active muscles. This comparison and its implications have already been fully discussed (Hill, 1953*b*; Hill & Howarth, 1959).

The values of the heat:tension ratio found in the present investigation were mostly near to 0·014. This is smaller than the value of 0·018 obtained by Hill (1953*b*). The present experiments are probably the more accurate because (*a*) the releases were studied during a continuing tetanus; (*b*) a quantitative allowance for the shortening heat has been made; and (*c*) the accurate method of synthesis has been used in the comparison of tension changes with heat deflexions. These differences in technique seem enough to account for the different values obtained.

If it is admitted that thermoelastic heat absorption accompanies the rise of tension following a rapid release, it seems most probable that a similar absorption of heat must occur during the original development of tension at the start of an isometric contraction, for the redevelopment of tension after a release follows a course so similar to that of the initial rise of tension (Hill, 1953*a*; Jewell & Wilkie, 1958). The effects of this heat absorption have been discussed by Hill & Howarth (1959). They point out how much simpler the time course of liberation of energy in an isometric twitch appears when due account is taken of the thermoelastic effect. The value of the thermoelastic heat:tension ratio used in their calculation ($-0{\cdot}018$) is probably rather too high, but the value varies so much from one muscle to another that changing the value to $-0{\cdot}014$ would not make the calculations any more precise. But it is perhaps worth re-emphasizing here how complex the heat production is in the apparently simple isometric twitch. Even if it is assumed that the muscle behaves uniformly, i.e. one part does not stretch another, there are four separate causes of heat production which combine to give the observed pattern. They are (1) the positive activation heat, (2) the positive shortening heat produced as the contractile component shortens against the series elastic elements, (3) the thermoelastic heat, negative during rise of tension and positive during fall of tension, and (4) the degradation of mechanical potential energy producing extra heat during relaxation.

SUMMARY

1. Experiments have been made on toad sartorius muscles to find out whether rise of tension in active muscle is accompanied by an absorption of heat similar to the production of heat known to accompany fall of tension.

2. The spontaneous redevelopment of tension following a quick release is accompanied by an absorption of heat which is revealed by comparison of the recorded heat production with an isometric control.

3. When the extra heat deflexion following a release is corrected for the shortening heat produced as the tension was redeveloped, its time course is found to be very similar to that of the thermoelastic heat deflexion calculated from the recorded tension changes. The assumption made in the calculation, that any tension change ($\Delta P$) is accompanied by a proportional heat change ($\Delta Q$), is therefore confirmed. The relation found was

$$\Delta Q = -0{\cdot}014 . l_0 . \Delta P,$$

although the numerical constant (thermoelastic heat : tension ratio) varied widely.

4. The thermoelastic heat : tension ratio is independent of the amount of release, and is also independent of the speed of release, if a correction is made for the heat produced by a viscous resistance to movement of the muscle of 500 dyn/(cm/sec). The viscous resistance was measured and found to be of this order.

5. Experiments are described in which a muscle contracting isotonically is stretched sufficiently to make the tension rise almost to the maximum isometric value, and the heat production is compared with that during an uninterrupted isotonic contraction. Only for stretches at less than 10 mm/sec was the heat production during the stretch less than in the isotonic control. In these slow stretches the difference from the isotonic control was often more than could be accounted for by the difference of shortening heat production. This heat absorption cannot, however, be positively identified as thermoelastic.

6. When a muscle is restretched to its initial length immediately after a quick release, extra heat appears which masks any absorption of thermoelastic heat. This extra heat production is partly due to a spontaneous fall of tension which follows a release-and-stretch.

7. The accuracy of the identification of the thermoelastic heat absorption found during spontaneous tension redevelopment after a quick release is discussed.

I should like to thank Professor A. V. Hill, under whose direction this work was carried out, for his constant guidance and encouragement. I am also grateful to Dr J. Délèze for

his help with the mechanical experiments on viscous resistance, and to Dr D. R. Wilkie for much valuable advice. The work was done during tenure of a M.R.C. scholarship for training in research methods.

## REFERENCES

ABBOTT, B. C. (1951). The heat production associated with the maintenance of a prolonged contraction and the extra heat produced during large shortening. *J. Physiol.* **112**, 438–445.

ABBOTT, B. C. & AUBERT, X. M. (1951). Changes of energy in a muscle during very slow stretches. *Proc. Roy. Soc.* B, **139**, 104–117.

ABBOTT, B. C., AUBERT, X. M. & HILL, A. V. (1951). The absorption of work by a muscle stretched during a single twitch or short tetanus. *Proc. Roy. Soc.* B, **139**, 86–104.

AUBERT, X. M. (1956). *Le couplage énergétique de la contraction musculaire.* Thèse d'agrégation, Université Catholique de Louvain. Brussels: Editions Arcia.

HARTREE, W. (1929). The identity of the response of muscle to direct and indirect stimulation. *J. Physiol.* **67**, 372–376.

HARTREE, W. (1931). The analysis of the initial heat production of muscle. *J. Physiol.* **72**, 1–26.

HILL, A. V. (1938). The heat of shortening and the dynamic constants of muscle. *Proc. Roy. Soc.* B, **126**, 136–195.

HILL, A. V. (1949). The energetics of relaxation in a muscle twitch. *Proc. Roy. Soc.* B, **136**, 211–219.

HILL, A. V. (1952). The thermodynamics of elasticity in resting striated muscle. *Proc. Roy. Soc.* B, **139**, 464–497.

HILL, A. V. (1953*a*). The mechanics of active muscle. *Proc. Roy. Soc.* B, **141**, 104–117.

HILL, A. V. (1953*b*). The 'instantaneous' elasticity of active muscle. *Proc. Roy. Soc.* B, **141**, 161–178.

HILL, A. V. (1953*c*). A reinvestigation of two critical points in the energetics of muscular contraction. *Proc. Roy. Soc.* B, **141**, 503–510.

HILL, A. V. & HOWARTH, J. V. (1957). Alternating relaxation heat in muscle twitches. *J. Physiol.* **139**, 466–473.

HILL, A. V. & HOWARTH, J. V. (1959). The reversal of chemical reactions in contracting muscle during an applied stretch. *Proc. Roy. Soc.* B, **151**, 169–193.

JEWELL, B. R. (1959). *The mechanical properties of muscle during contraction and relaxation.* Ph.D. Thesis, University of London.

JEWELL, B. R. (1960). The nature of the phasic and the tonic responses of the anterior byssal retractor muscle of *Mytilus*. *J. Physiol.* **149**, 154–177.

JEWELL, B. R. & WILKIE, D. R. (1958). An analysis of the mechanical components in frog's striated muscle. *J. Physiol.* **143**, 515–540.

PROCEEDINGS OF
THE PHYSIOLOGICAL SOCIETY

MILL HILL MEETING

4–5 *November* 1960

## Push-pull cannulae

By J. H. Gaddum. *A.R.C. Institute of Animal Physiology, Babraham, Cambridge*

The object of these cannulae is to collect chemical transmitters and other substances liberated in the tissues. They are based on a technique used by Fox & Hilton (1958) who perfused the subcutaneous tissues between two parallel needles 1·5–2 cm apart.

A suitable fluid such as Locke's solution runs from a high reservoir through a fine tube into living tissue; it is collected by another tube surrounding the tube through which it arrives. These tubes may both be steel needles, or one of them may be polythene. The rate of inflow is measured by the formation of drops, and adjusted by a suitable tap to about 0·1–0·2 ml./min. The apparatus is first tested in air. The rate of outflow depends on the difference in height ($h$) between the needle and the lower end of the outflow tube. The outflow tube is raised (so that $h$ is small) and the tube filled with liquid from below; drops fall both from the needle and from the outflow. The value of $h$ is then increased, and, as the outflow increases, fewer drops fall from the needle. Over a certain range of values of $h$, no drops fall from the needle and inflow and outflow are equal; the apparatus is then ready for use. It works equally well whether the needle is pointing up or down or horizontally. When $h$ is increased still further, air is sucked into the outflow tube, and the siphon is broken. This does not occur when the needle is in a tissue, and it may be desirable in some cases to alter $h$ until satisfactory results are obtained.

The apparatus can be used in tissues receiving their normal supply of blood, and, although some blood may appear in the effluent when the needle is moved, the fluid has been found to become clear in a short time. The effluent has been tested in a microbath not unlike that described by Gaddum & Stephenson (1958); acetylcholine and other pharmacologically active substances have been detected. It seems likely that these substances have reached the flowing solution by diffusion.

Theoretically this apparatus has the following advantages:

(1) It detects the substances liberated under fairly normal conditions.

(2) It gives an indication of the turnover rather than the store, and can be used to study the effect of factors such as nervous stimulation.

(3) It should be possible to localize the site of liberation of substances in such tissues as the central nervous system more precisely than by other means.

(4) The blood–brain barrier presumably does not intervene.

REFERENCES

Fox, R. H. & Hilton, S. M. (1958). *J. Physiol.* **142**, 219.
Gaddum, J. H. & Stephenson, R. P. (1958). *Brit. J. Pharmacol.* **13**, 493.

## A vacuum bottle sampler for expired air

By B. M. Wright. *National Institute for Medical Research, Mill Hill, London, N.W.7*

This sampler has been designed for use with a respiratory anemometer (Wright, 1955) but is suitable for use with any form of gas meter, such as the Kofranyi–Michaelis Respirometer (Kofranyi & Michaelis, 1940) or the Integrating Motor Pneumotachograph (Wolff, 1958), that can make an electrical contact every time a certain volume of gas has passed through it. The sampler can also be used for end-tidal air sampling when connected to a suitable respiratory switch.

The vacuum is contained in a 10 oz. (0·28 l.) flat medicine bottle fitted with a butyl rubber cork and two stainless-steel tubes so that mercury can be introduced to displace the sample. Butyl rubber tubing connects the bottle to the sampler, which consists of a combined reducing and electromagnetic valve.

The reducing valve brings the vacuum in the bottle to a constant suction of 15 cm Hg, and the electromagnetic valve is energized by a condenser discharge to give a constant opening time. Thus a sample of constant volume is aspirated each time a contact is made. This volume can be varied from 0·1 to 2·0 ml. by means of a calibrated valve.

The available volume in the bottle is about 240 ml. and the dead space in the sampler is about 1·0 ml. The vacuum in the bottle is indicated by two capillary tubes containing mercury beads which move at 1/100 and 4/5 atmosphere, respectively.

The weight of the bottle and sampler, including two 22½ V hearing-aid batteries, which will operate it for several days, is about 800 g.

A standard commercial Wright Respirometer is fitted with contacts to give a pulse every 1 l., and also with an internal bimetallic thermometer indicating from 0 to 40° C on the dial.

End-tidal air samples can be taken with either a specially designed belt switch or a simple switch fitted to a standard Siebe–Gorman valve.

REFERENCES

KOFRANYI, E. & MICHAELIS, H. J. (1940). *Arbeitsphysiologie*, **11**, 148.
WOLFF, H. S. (1958). *Quart. J. exp. Physiol.* **43**, 270.
WRIGHT, B. M. (1955). *J. Physiol.* **127**, 25*P*.

## Transistorized drop-counter with simple drop chamber for measurement of blood flow

By A. GOAD, N. L. GREGORY and J. MCKAY. *National Institute for Medical Research, Mill Hill, London, N.W. 7*

## Sedation and nystagmus produced by small doses of sodium barbital injected into the cerebral ventricles of rabbits

By A. HASSELBLATT and D. H. SPROULL. *National Institute for Medical Research, Mill Hill, London, N.W. 7*

## A lever-operated, spring-loaded plunger clip for organ-bath drain pipes

By A. R. J. COLLINS and J. P. B. STEAN. *National Institute for Medical Research, Mill Hill, London, N.W. 7*

## The transmission of serum proteins from foetus to mother in the Rhesus monkey: indwelling cannulation of the foetus without interruption of pregnancy

By D. R. BANGHAM, K. R. HOBBS and D. E. H. TEE. *National Institute for Medical Research, Mill Hill, London, N.W. 7*

## Further observations on brief profound anoxia

By J. ERNSTING, J. L. GEDYE and G. J. R. MCHARDY. *Royal Air Force Institute of Aviation Medicine, Farnborough, Hants*

We have described (Ernsting & McHardy, 1960) a method of producing brief anoxia by supplying oxygen at various times after decompression from 8000 to 38,000 ft. (560–155 mm Hg absolute) in 1·5 sec. In the present experiments the performance of six subjects on a task requiring the reproduction of a learned sequence of eight digital operations was assessed every 5 sec by measuring the time taken to complete the sequence; a four-channel electroencephalogram was also recorded. Each subject was

decompressed three times under each of three conditions, viz.: oxygen breathed before and after decompression (curves *A*, Fig. 1), and oxygen supplied 2 sec (curves *B*), and 8 sec (curves *C*) after decompression, air being breathed beforehand.

When oxygen was breathed throughout, no decrement in performance or change in e.e.g. was found. When oxygen was supplied at 2 sec after decompression performance was impaired for 15 sec. In addition, fast (8–13 c/s) activity was noted in the e.e.g. beginning at 15 sec and lasting until 25 sec after decompression. When oxygen was supplied 8 sec after

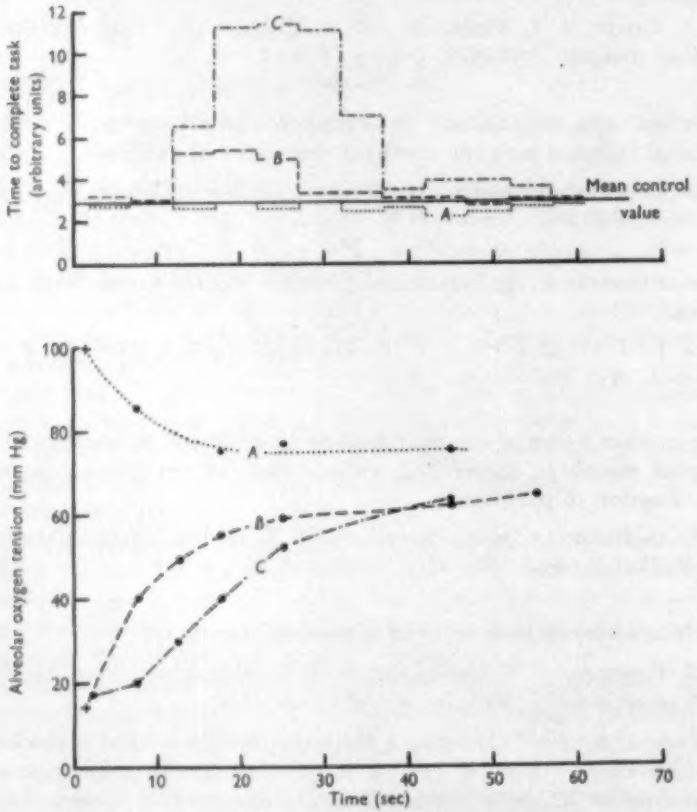



**Fig. 1.** Mean alveolar oxygen tensions and performance on task following rapid decompression from 8000 to 38,000 ft. in 1·5 sec. Curve *A*—oxygen breathed before and after decompression; curve *B*—air breathed before and oxygen supplied 2 sec after decompression; curve *C*—air breathed before and oxygen supplied 8 sec after decompression.

decompression there was gross impairment of performance, some subjects waiting for a considerable period before attempting the task and one losing consciousness. The e.e.g. showed changes which included the appearance or accentuation of 8–13 c/s activity and in some cases the appearance of slow (2–4 c/s) activity. These changes began some 15 sec after decompression and had disappeared by 30 sec. The delay in the appearance of e.e.g. changes can be attributed to the sum of the lung-to-brain circulation time, and the time required for the development of cerebral anoxia. It is of interest that the decrement in performance persisted for 20 sec after the e.e.g. changes had disappeared.

Figure 1 shows the changes in performance together with the mean values for alveolar oxygen tension derived from the earlier experiments.

When oxygen was supplied 2 sec after decompression the subject's first breath was pure oxygen. The subsequent rise in alveolar oxygen tension (curve *B*) represents the washing out of nitrogen from the alveolar gas. The brief anoxia occurring while the oxygen tension was low was none the less sufficient to impair performance, and cause changes in the e.e.g.

REFERENCE

Ernsting, J. & McHardy, G. J. R. (1960). *J. Physiol.* **153**, 73*P*.

## Neurogenic vasodilation and willed movement

By A. C. Dornhorst. *St George's Hospital Medical School, London, S.W.* 1

## The separation of the components of the menstrual stimulant

By H. J. Clitheroe* and V. R. Pickles.* *Department of Experimental Medicine, University of Cambridge*

The menstrual stimulant is a group of smooth-muscle stimulants which originate in the endometrium, and are thought to stimulate the myometrial contractions expelling the decidua menstrualis (Pickles, 1959). There have previously been known to be at least two stimulant substances in the group, but they were not clearly separated. We have now been able to separate *three* such stimulant components both from one another and from most of the inactive material in the crude lipid extracts. In addition to these, and clearly separable from them, there is found in many extracts a stimulant of non-menstrual origin, of which an oral account was given at the Society's meeting of 22–23 April 1960.

Until they can be chemically identified, these components of the menstrual stimulant are referred to as *A*, *B*, and *C*. The following are the principal methods used for their separation.

* Present address: Physiology Department, University of Sheffield.

(1) On partition of crude lipid extracts (acetone- and dichloromethane-soluble material) between heptane and 80% aqueous methanol, *C* remains largely in the heptane while *B* and *A* largely pass into the methanol.

(2) Fractional elution from silica gel enables the stimulants to be further separated. Thus in the heptane fraction from (1) above, *C* is eluted by 10–15% ether in petrol but not by 5%. Likewise in the methanol fraction, *B* is eluted by 15% ether in petrol, and *A* by 10–30% methanol in ether. Intermediate fractions include the main non-menstrual stimulant.

(3) Components *A* and *B* can be passed from solution in ether or dichloromethane into $NaHCO_3$ solution, and back into an organic solvent after acidification of the aqueous phase; *C* however remains in the first organic phase.

(4) *C* can then be separated from much inactive material by fractional elution from partially hydrated alumina.

(5) *B*, prepared by methods (1), (2) and (3), can be further purified by chromatography on a column of kieselguhr, from which it is very readily eluted by petrol.

(6) *A* can be further purified by chromatography on a cellulose-powder column developed with an acetone–ether mixture.

(7) Paper chromatography (ethyl methyl ketone–diethylamine–water 60:3:20; Vogt, 1957) has been used in supplementary tests of purity, but is unsuitable for preparative purposes.

These three components have all the effects on smooth muscle that have been described before for the menstrual stimulant, while the discarded lipid (90% of the total weight) is of much smaller stimulant potency. All the components are 'slowly acting' by comparison with histamine or ACh; but *C* has particularly slow and persistent effects. *B* is the most effective potentiator of other stimulants, and *A* is the most potent in direct stimulation of the guinea-pig uterus.

REFERENCES

Pickles, V. R. (1959). *J. Endocrin.* **19**, 150.
Vogt, W. (1957). *J. Physiol.* **137**, 154.

## Dark adaptation of human rods and the regeneration of rhodopsin

By W. A. H. Rushton. *Department of Ophthalmology, National Institutes of Health, Bethesda, Md., U.S.A.*

Hecht's well-known photochemical theory did not treat quantitatively the increase of sensitivity which occurs during dark adaptation, nor was the postulated regeneration of rhodopsin ever measured. Such measurements are now possible in the human eye by the technique of retinal

densitometry, so the relation of threshold to the amount of pigment regenerated may be found by direct determination.

The subject was exposed to a bright light which bleached more than 25% of his rhodopsin in the exposed area. Regeneration was then observed by densitometer readings. When 85% had regenerated the subject shifted to a conventional dark-adaptation apparatus and the threshold was always found still to lie upon the cone branch of the curve. A minute or two later the kink occurred and the instant of cone–rod transition was noted. The subject returned to the densitometer and completed the rhodopsin curve. By interpolation it was easy to read that the kink occurred when 92% of rhodopsin was in the rods, and this figure was found no matter how extensive was the initial bleaching. Whenever only 8% remained unregenerated, the rods entered (at 2 log. units above the final threshold), and the smaller the initial bleach, the earlier this moment occurred.

In normal eyes the relation between rhodopsin and rod threshold can only be studied for the last 10% of regeneration, since greater bleaching makes rods less sensitive than cones. But a larger range is available in rod monochromats where cones are practically absent (work in collaboration with M. G. F. Fuortes and R. D. Gunkel).

The monochromat's rhodopsin was found to be normal in amount and in regeneration rate, and the rod dark adaptation could be followed over a range of 6 log. units. The relation between the fully dark threshold $I_0$ and the threshold $I$ at the moment when a fraction $p$ of rhodopsin was still unregenerated is given by

$$\log (I/I_0) = 20p.$$

This relation is similar to that found recently in the rat by Dowling & Wald (1960) between the threshold for the e.r.g. and the amount of rhodopsin extracted at various stages of dark adaptation or of vitamin A deprivation.

REFERENCE

DOWLING, J. E. & WALD, G. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 587–603.

## Pulsatile gas flow in bronchi caused by the heart beat

By P. HUGH-JONES and J. B. WEST. *Department of Medicine, Postgraduate Medical School, Hammersmith Hospital, London, W.*12

A new type of flowmeter allows measurement of the gas flow rate in small bronchi at bronchoscopy. The principle is that a fine jet of an inert gas is blown across a small gap between two opposing fine tubes. One tube delivers the gas, and the other is connected to a rapid gas analyser. When the flowmeter, protected by a small wire cage, is placed in a bronchus

so that the jet is at right angles to the direction of bronchial gas flow, the inert gas stream is deflected, so that the concentration recorded by the analyser is a measure of bronchial flow rate (West, 1960).

By using this flow meter, pulsatile gas flow synchronous with the heart beat has been recorded in segmental and lobar bronchi in man. Pulsations were detected in three-quarters of fifty-four patients examined and occurred in all parts of the lung, though particularly on the left side. They were seen during both inspiration and expiration but were most obvious in the post-expiratory pauses. Volume flow rates up to 2 l./min and displaced volumes up to 5 ml. were recorded. At a given instant of the cardiac cycle, the phase of the cardiac pulsations was sometimes different in different parts of the lung. The pulsations are presumably caused by movements of the heart compressing some parts of the lung and allowing others to expand.

REFERENCE

WEST, J. B. (1960). Measurement of bronchial air flow. *J. appl. Physiol.* **15**, 976.

## The release of serotonin by intravenous adrenaline and noradrenaline

By T. M. ANDREWS, W. S. PEART and J. I. S. ROBERTSON. *Medical Unit, St Mary's Hospital, London, W.2*

Attacks of facial flushing in patients with metastatic carcinoid tumours can be provoked by the intravenous injection of small doses of adrenaline and noradrenaline, probably by the release of a humoral substance from the neoplasm (Peart, Robertson & Andrews, 1959). Serotonin does not appear to be the substance responsible for the flush, though it remains possible that serotonin release from the tumour could occur in some of these cases during the flushing attack.

The subject of the present study was a 58-year-old woman with a primary carcinoid tumour of the ileum and secondary deposits in the liver. Rapid serial blood samples were obtained by cardiac catheter, variously from an hepatic vein and from the upper inferior vena cava during flushing attacks provoked by the intravenous injection of 2 $\mu$g of adrenaline and 10 $\mu$g of noradrenaline. Free plasma serotonin was assayed in these samples on the fundal strip preparation of the rat's stomach (Vane, 1957), using the technique to be published by Robertson and Andrews. In all the attacks, a rise in the free plasma serotonin occurred from basal levels of 20 to 40 ng/ml. to peak levels varying from 320 to 1000 ng/ml.

This observation adds another example to the group of interrelationships which are known to exist between various amines present in the body. Other instances are the release of adrenaline and noradrenaline from adrenal

medullary tumours by intravenous histamine (Roth & Kvale, 1945), the suggested release of adrenaline from the adrenal medulla of the cat by serotonin (Reid, 1952) and the release of histamine by serotonin (Feldberg & Smith, 1953).

Of interest in the present context is the observation that the distribution of serotonin in the brain closely follows the noradrenaline content (Amin, Crawford & Gaddum, 1954). The precise significance of these various relationships remains to be determined.

REFERENCES

Amin, A. H., Crawford, T. B. B. & Gaddum, J. H. (1954). *J. Physiol.* **126**, 596.
Feldberg, W. & Smith, A. N. (1954). *Brit. J. Pharmacol.* **8**, 406.
Peart, W. S., Robertson, J. I. S. & Andrews, T. M. (1959). *Lancet*, **277**, 715.
Reid, G. (1952). *J. Physiol.* **118**, 435.
Roth, G. M. & Kvale, W. F. (1945). *J. Lab. clin. Med.* **30**, 366.
Vane, J. R. (1957). *Brit. J. Pharmacol.* **12**, 344.

## The effect of nitrate on electrical and mechanical activity of smooth muscle

By J. Axelsson. *Department of Pharmacology, University of Oxford*

Hill & Macpherson (1954*a*, *b*) found that $NO_3$ increased the tension response to single shocks in frog's striated muscle. They suggested it did so by extending in duration some process in the membrane.

The present experiments were carried out on intestinal smooth muscle with simultaneous recording of mechanical and electrical activity by the sucrose-gap method.

When the muscle was stimulated electrically at a frequency somewhat higher than its spontaneous rate, it responded to each stimulus by one action potential. When the Cl was replaced by $NO_3$ each stimulus evoked two or three action potentials and consequently the tension was greatly increased by summation. A single stimulus now caused repetitive discharge. During spontaneous activity, replacement of Cl by $NO_3$ was found to double or treble the rate of discharge. It appeared as if, in smooth muscle, the increased tension in $NO_3$ solution was a consequence of increased spike frequency. However, when a similar increase in frequency was obtained by raising the temperature, the increase in tension was not maintained. Moreover, if the spike frequency was already high because the driving frequency was rapid or because the temperature was raised, replacement of Cl with $NO_3$ caused no further increase in frequency of action potentials; nevertheless, each individual contraction was larger. This action of $NO_3$ could still have been secondary to a change in electrical

activity, since it was observed that the repolarization of the compound spike was delayed. The tail of the action potential was prolonged. An increased duration of the action potential in $NO_3$ solution has been recorded by intracellular electrodes in uterus muscle by H. Kuriyama (personal communication) and in frog skeletal muscle (Hutter & Noble, 1960), and by external electrodes by Harris (1958) in frog sartorius.

A correlation between membrane resistance and tension response in striated muscle has been suggested, as anions which increase membrane resistance have been found to increase the duration of active state (Hutter & Padsha, 1959; Hutter & Noble, 1960). Therefore, in a few experiments, Cl was replaced by ethanesulphonate to which the membrane is assumed to be even less permeable than to $NO_3$. Its effect was, however, not comparable to that of $NO_3$, although it usually increased temporarily the spike frequency.

Considering the possibility that $NO_3$ had a direct action on the contractile mechanism, we studied its action in conditions in which the tension was reduced without reduction in electrical activity (Axelsson & Bülbring, 1959; Axelsson, 1960). We found that $NO_3$ did not increase the tension response in Ca-free solution nor when Na had been replaced by Li, although spontaneous spike discharge was greatly increased.

REFERENCES

Axelsson, J. & Bülbring, E. (1959). *J. Physiol.* **149**, 50–51.
Axelsson, J. (1960). *J. Physiol.* **152**, 16–17.
Harris, E. J. (1958). *J. Physiol.* **141**, 351–365.
Hill, A. V. & Macpherson, L. (1954*a*). *J. Physiol.* **125**, 17*P*.
Hill, A. V. & Macpherson, L. (1954*b*). *Proc. Roy. Soc.* B, **143**, 81–102.
Hutter, O. F. & Padsha, S. M. (1959). *J. Physiol.* **146**, 117–132.
Hutter, O. F. & Noble, D. (1960). *J. Physiol.* **151**, 89–102.

## Interactions of acetylcholine, adrenaline and magnesium with calcium in the contraction of depolarized rat uterus

By K. A. P. Edman and H. O. Schild. *Department of Pharmacology, University College London*

Smooth muscle which has been depolarized by immersion in isotonic $K_2SO_4$-Ringer still responds to acetylcholine (ACh) (Evans, Schild & Thesleff, 1958). This response requires calcium, as was demonstrated by Robertson (1960) and Jenkinson (personal communication). We have found that calcium itself contracts the depolarized rat uterus. The threshold concentration for an isometric contraction at room temperature is 0·1 mM calcium. The response increases up to about 1·2 mM and remains constant or declines with higher concentrations (up to 4·8 mM). The greatest isometric

tensions by calcium in the depolarized preparation are about 50 % of the maximum tensions produced by ACh in normal Ringer's solution. The calcium contractions are maintained as long as calcium is present in the bath, but they are completely reversible by removing the calcium from the solution and can be repeated over hours. Magnesium (2–8 mM) produces a graded antagonism of the calcium contractions.

ACh applied to a depolarized rat uterus preparation which is already contracted by calcium produces a further contraction. Thus if the tension produced by calcium is 50 % of the maximum, ACh may increase this to 80–90 %. It is of interest that the effects of ACh cannot be duplicated by external application of calcium in view of the importance attributed to penetration of calcium into the muscle cell in the initiation of contraction (Shanes, 1958).

Adrenaline in low concentrations ($10^{-9}$ to $10^{-8}$) relaxes the uterus contracted by calcium and inhibits the calcium-induced contraction of a relaxed uterus.

Calcium is also required for the depolarization-contraction of rat uterus following immersion in isotonic $K_2SO_4$-Ringer in accordance with the findings reported by Niedergerke (1956) for heart muscle and Frank (1960) for striated muscle. If a rat uterus which is kept in calcium-free NaCl-Ringer is briefly immersed at intervals in isotonic calcium-free $K_2SO_4$-Ringer the depolarization-contractions thus elicited gradually diminish and eventually disappear. The responses to ACh in the calcium-free NaCl-Ringer also decline but at a slower rate, so that a stage can be reached where immersion in $K_2SO_4$-Ringer produces no tension response but ACh still produces a considerable effect. This is a further argument in favour of the view that ACh contractions of smooth muscle can be produced by a mechanism other than a depolarization.

## REFERENCES

Evans, D. H. L., Schild, H. O. & Thesleff, S. (1958). *J. Physiol.* **143**, 474–485.
Frank, G. B. (1960). *J. Physiol.* **151**, 518–538.
Niedergerke, R. (1956). *J. Physiol.* **134**, 584–599.
Robertson, P. A. (1960). *Nature, Lond.*, **186**, 316–317.
Shanes, A. M. (1958). *Pharmacol. Rev.* **10**, 165–273.

## Enhancement of the anaphylactic release of histamine and slow-reacting substance by certain dibasic fatty acids and inhibition by monobasic fatty acids

By K. F. AUSTEN* and W. E. BROCKLEHURST.† *National Institute for Medical Research, Mill Hill, London, N.W.7*

The amounts of both histamine and slow-reacting substance 'SRS-A' (Brocklehurst, 1960) released from perfused, chopped, guinea-pig lung during anaphylaxis *in vitro* are greatly increased by the presence of succinic acid. In seventeen different experiments an average of 19·5% of the total tissue histamine was released by antigen alone, whereas 37·5% was released when 0·5 mM succinic acid was present in the reaction mixture. This enhancement occurred when the antigen was added to the sensitized tissue 10 sec after the suspending medium had been changed from normal Tyrode solution to Tyrode containing succinic acid. Incubation of the tissue with succinic acid for 5 min before adding antigen produced only one-third more enhancement than the 10 sec pre-incubation, while pre-incubation for 15 and 30 min gave no further increase. Enhancement of the anaphylactic reaction was definite with as little as 0·05 mM succinic acid and concentrations of 5·0 and 20·0 mM were only slightly more effective than 0·5 mM. Succinic acid alone did not release detectable quantities of histamine and SRS-A.

No enhancement was achieved with Krebs's cycle intermediates such as citric acid, fumaric acid, and malic acid, but α-keto glutaric acid at 5·0 mM was almost as active as succinic acid at 0·5 mM. Other dibasic acids such as malonic, adipic, and pimelic were virtually inactive, while glutaric had approximately the same activity as α-keto glutaric. In contrast to fumaric acid (trans), which was inactive even at 20 mM, maleic acid, the cis isomer, was as active at 0·5 mM as succinic acid. Thus, the essential chemical structure for enhancement of the anaphylactic reaction in guinea-pig lung appears to be a dibasic acid in which the carboxyl groups have a cis configuration and are separated by a two carbon chain.

Monobasic acids, on the other hand, inhibit the anaphylactic release of histamine and SRS-A, and the concentration required to produce 50% inhibition decreases with increasing chain length. Thus, 0·5 mM decanoic acid produces the same inhibition as 5·0 mM of hexanoic acid. This inhibition is not reversed by excess calcium, and so cannot be due to the removal of calcium. Substitution of hexanoic acid with an ε-amino group diminishes the inhibitory capacity, and substitution with an α-amino group abolishes it.

* U.S.P.H.S. Postdoctoral Research Fellow.

† Present address: Department of Pharmacology, University New Buildings, Teviot Place, Edinburgh, 8.

The mechanism whereby the monobasic fatty acids from pentanoic to dodecanoic inhibit and the four carbon dibasic acids, succinic and maleic, enhance the anaphylactic release of histamine and SRS-A is not established. It is possible, however, that they are competing for the same site in the reaction sequence.

These studies show that compounds normally existent in mammalian tissue can respectively enhance or inhibit the *in vitro* anaphylactic reaction in chopped guinea-pig lung.

REFERENCE

BROCKLEHURST, W. E. (1960). *J. Physiol.* **151**, 416–435.

## Visceral receptors, pain, bradykinin, and analgesic agents

By C. BRAUN, F. GUZMAN, E. W. HORTON, R. K. S. LIM and G. D. POTTER. *Medical Sciences Research Laboratory, Miles Laboratories, Inc., Elkhart, Indiana, U.S.A.*

Pericapillary non-myelinated fibres and branching varicose terminals course alongside, but do not end upon, the capillary wall (methylene blue and silver methods in rat and cat). These pericapillary terminals appear similar to the free-branching 'pain' receptors in cutaneous epithelium (Weddell & Pallie, 1954; Lim, 1960).

The perivascular network in larger vessels containing one or more layers of muscle consists largely of non-myelinated sympathetic efferents, which disappear after sympathectomy. The remaining fibres are derived from myelinated axons and are sensory fibres arising from spinal ganglia. Some of these terminate as pericapillary receptors of the *vasa vasorum* (and *vasa nervorum*).

Intra-arterial injection of KCl (0·5 ml., 18·75 mg), bradykinin* (0·5 ml. = 1·2 $\mu$g pure bradykinin) or acetylcholine (0·5 ml., 0·5 mg) in the chloralose dog (80 mg/kg I.V.) evokes a response consisting of vocalization, hyperpnoea and vasomotor change, which disappears on section of the nerves to the organ or part supplied by the injected vessels. On the other hand, intravenous and caval injections are without effect. Intra-arterial injections into the leg after removal of the skin, or into the splenic, gastric, intestinal or internal carotid arteries, or portal vein, all give the response.

Excluding KCl as a general depolarizing agent which may excite axons, and assuming that the other agents excite receptors (Diamond, 1959), it is evident that they are capable of exciting both the cutaneous and the visceral (pericapillary) receptors, which evoke a common response.

* Crude bradykinin used was from a sample kindly provided by Drs D. F. Elliott and G. P. Lewis and from other batches prepared in this laboratory.

In view of the fact that bradykinin is the most effective agent and produces pain in man (Armstrong, Keele, Jepson & Stewart, 1954), it may well be one of the agents responsible for the causation of visceral pain under different conditions of inflammation or internal nociception. Rocha e Silva & Antonio (1960) report that thermal injury to the rat's paw liberates bradykinin into the warmed tissues. These observations support the idea that bradykinin acts as a mediator of inflammatory changes (Elliott, Horton & Lewis, 1960).

Preliminary tests have shown that the intravenous administration of sodium acetylsalicylate (ASA) 25–50 mg/kg and *N*-acetyl-*p*-aminophenol (APAP) 10–20 mg/kg may block the response to bradykinin, etc., completely (ASA 4/11, APAP 2/8 dogs), or partially (ASA 5/11, APAP 1/8 dogs), and that morphine sulphate 1·0 mg/kg is the most powerful blocking agent (6/7 dogs). At these dose levels, the response begins to recover in 45–60 min.

These observations suggest that if the features of the response, particularly vocalization, are indicators of visceral pain, the latter may be produced by the action of bradykinin on visceral (pericapillary) receptors and reduced by the action of non-narcotic and narcotic analgesics.

REFERENCES

Armstrong, D., Keele, C. A., Jepson, J. B. & Stewart, J. W. (1954). *Nature, Lond.*, **174**, 791.

Diamond, J. (1959). *J. Physiol.* **145**, 611.

Elliott, D. F., Horton, E. W. & Lewis, G. P. (1960). *J. Physiol.* **153**, 473.

Lim, R. K. S. (1960). *Ann. N.Y. Acad. Sci.* **86**, 73.

Rocha e Silva, M. & Antonio, A. (1960). *Pharmacologist*, **2**, 89.

Weddell, G. & Pallie, W. (1954). Peripheral circulation in man. *Ciba Found. Symp.* p. 132. London: Churchill.

## The effect of adrenaline, noradrenaline and isoprenaline on the guinea-pig's uterus

By K. Hermansen*. *Department of Pharmacology, University of Oxford*

Uteri from immature guinea-pigs weighing about 250 g were set up in an isolated organ bath containing 20 ml. of a modified Krebs's solution ((mM) Na 137; K 5·9; Ca 2·5; Mg 1·2; Cl 135; $HCO_3$ 15·0; $H_2PO_4$ 1·2; glucose 11·5; 97% $O_2$; 3% $CO_2$, pH 7·3, 35° C) with a tension of 2 g.

Doses of adrenaline (1–20 $\mu$g), noradrenaline (0·1–4 $\mu$g) and isoprenaline (0·1–20 $\mu$g) were given repeatedly during periods up to 8 hr. Noradrenaline always caused a contraction. Isoprenaline always caused a relaxation when a tone was present, but had no visible effect when the uterus was atonic.

* On leave from Pharmacia, Ltd., Vanlose, Copenhagen, Denmark.

Adrenaline had a complex effect:

(*a*) *When no tone was present* adrenaline had no visible effect at the beginning of an experiment but at the end it caused a contraction.

(*b*) *When a moderate spontaneous tone was present* the effect of adrenaline at the beginning was a relaxation. It gradually changed to a biphasic effect (contraction followed by relaxation) and finally to a contraction.

(*c*) *When a high tone was present*, which occurred sometimes spontaneously at the end of an experiment, or, when it was produced by noradrenaline or acetylcholine, adrenaline caused relaxation.

Isoprenaline abolished the relaxing effect of adrenaline. If, in the presence of isoprenaline, a high tone was produced by acetylcholine, adrenaline caused a further contraction. If, on the other hand, a high tone was produced by noradrenaline, adrenaline caused neither contraction nor relaxation.

Adrenaline had a pure motor effect on uteri from guinea-pigs treated with oestrogens, as already demonstrated by Holtz & Wölpert (1937) and Greeff & Holtz (1951).

The motor effect of adrenaline was always enhanced by isoprenaline in uteri from both immature and oestrogen-treated guinea-pigs.

After the experiments the uteri were taken for Na and K determinations and the results were:

| | Na | K | |
|---|---|---|---|
| Immediately after killing | 71·5 ± 12·0 | 80·2 ± 6·6 | mM/kg fresh wt. |
| After 8 hr expt. | 70·8 ± 7·0 | 77·8 ± 12·4 | mM/kg wet wt. |

As the water content did not change during an experiment, it seems that the Na and K concentrations remained constant after 8 hr repeated application of the amines.

The glycogen content was determined in uteri kept for 8 hr in the same experimental conditions as above. There was a gradual fall to 50 % of the initial value in 4 hr but no further decline up to 8 hr.

The relaxing effect of adrenaline on intestinal smooth muscle has been interpreted as the result of an increased rate of supply of metabolic energy from glycogenolysis, as adrenaline causes an increase in phosphorylase activity (Axelsson, Bueding & Bülbring, 1959; Axelsson & Bülbring, 1960). If a similar mode of action is assumed for uterine muscle some of the observations described in this communication might be explained.

## REFERENCES

Axelsson, J., Bueding, E. & Bülbring, E. (1959). *J. Physiol.* **148**, 62–63*P*.
Axelsson, J. & Bülbring, E. (1960). *J. Physiol.* **153**, 30*P*.
Greeff, K. & Holtz, P. (1951). *Arch. int. Pharmacodyn.* **88**, 228.
Holtz, P. & Wölpert, K. (1937). *Arch. exp. Path. Pharmak.* **185**, 20.

## Studies with histidine decarboxylase

By J. M. Telford and G. B. West. *Department of Pharmacology, School of Pharmacy, University of London*

The presence of histidine decarboxylase, the enzyme responsible for the formation of histamine from histidine, has been investigated by many workers, but few have studied the factors affecting the *in vitro* determination of this enzyme. Using the method of Waton (1956), we have now investigated some of these factors which modify the activity of histidine decarboxylase in rat tissues.

With the optimal conditions of incubation for each tissue, the chief sources of histidine decarboxylase in the adult rat were pyloric stomach (where more than 8 $\mu$g histamine/g tissue per hour was formed), the liver (5·1 $\mu$g/g), the duodenum (3·3 $\mu$g/g) and the kidney (1·6 $\mu$g/g). These results are in agreement with those found by previous workers, except for those found for the liver. In this tissue, the high activity was only obtained when incubation proceeded at a pH value of 8·0 in the presence of traces of benzene, and this may account for the low activity found by previous workers.

According to Kahlson (1960), histidine decarboxylase in rat liver undergoes dramatic increases in activity during active growth, as, for example, in the development of the foetus and in the adult after partial hepatectomy. We tested the histidine decarboxylase in foetal rat liver using Waton's method and confirmed the high activity. For example, the histamine formed by liver extracts from foetuses of 14–16 days ranged from 500 to 1250 $\mu$g/g tissue per hour. These values, however, were obtained when incubation proceeded at a pH value of 6·5 and there was much less activity at pH 8·0. Furthermore, traces of benzene in the incubation mixture failed to modify the enzyme activity. The change-over in the optimal pH value from 6·5 to 8·0 was detected just after birth, when the enzyme activity fell to near the adult level.

It has not been possible so far to confirm the high histidine decarboxylase activity of adult rat liver after partial hepatectomy. Further work is also in progress to determine whether glucocorticoids influence the histidine decarboxylase activity of rat tissues.

### REFERENCES

Kahlson, G. (1960). *Lancet*, **278**, 67–71.
Waton, N. G. (1956). *Brit. J. Pharmacol.* **11**, 119–127.

## Electrical potentials in the isolated intestine

By R. J. C. Barry, S. Dikstein, J. Matthews and D. H. Smyth. *Department of Physiology, University of Sheffield*

As everted sacs of rat intestine are now widely used to study intestinal transport, and as transfer of ions in many tissues is associated with electrical potentials, it was of interest to see whether electrical potentials could be recorded in everted sacs.

White rats were anaesthetized with pentobarbitone and sacs were made from the middle fifth of the intestine. Potentials were led off by agar bridges to calomel electrodes connected with a Pye portable potentiometer. One of the bridges ended inside the sac and the other was in contact with the fluid in which the sac was suspended. The sacs were filled with, and suspended in Krebs's bicarbonate saline with or without glucose. When glucose was present (500 mg/100 ml.) a steady potential of about 8 mV was recorded, the serosal side being positive to the mucosal side, i.e. the positive potential was on the side to which sodium transfer takes place. If glucose was absent a much smaller potential was recorded, or in some cases no potential or a small potential in the opposite direction. The glucose-dependent potential was dramatically reduced by addition of phlorrhizin ($10^{-5}$ to $10^{-3}$ M) to the mucosal fluid. The initial effect of phlorrhizin was very rapid and a fall of about 5 mV had occurred in the few seconds necessary to make a measurement after addition of phlorrhizin. The phlorrhizin effect was partially reversible on replacement of the mucosal solution by a phlorrhizin-free solution. Phlorrhizin had no effect in the absence of glucose. The potential was considerably reduced, but not abolished, by addition of DNP ($10^{-4}$ M) to the mucosal fluid. The potential was also reduced but not abolished by replacing the oxygen bubbling through the mucosal fluid with nitrogen. Provided the anoxia lasted only a short time, the initial potential was obtained on readmission of oxygen.

Curran & Solomon (1957) have recorded a potential in the ileum of the rat *in vivo*. In this case the mucosal side was positive and their interpretation was that more anions were transported from the lumen than cations. If the potential in our experiments is due to sodium, a more rapid transfer of cations from the lumen is suggested. Newey, Parsons & Smyth (1959) have shown that the entry of glucose into the mucosal cells is prevented by phlorrhizin, and the present findings would suggest that phlorrhizin affects sodium entry, not necessarily directly, but possibly by preventing glucose entry. This is of interest in relation to the suggestion of Crane, Miller & Bihler (1961) that there is a direct relationship between sodium transfer and glucose transfer by the intestine. Barry, Matthews & Smyth (1959) have shown that there are two types of water transfer by the intes-

tine, one dependent on glucose and one independent of glucose, and the potentials which we described here are possibly related to the glucose-dependent water transfer.

REFERENCES

BARRY, B. A., MATTHEWS, J. & SMYTH, D. H. (1959). *J. Physiol.* **149**, 78–79*P*.

CRANE, R. K., MILLER, D. & BIHLER, I. (1961). *Membrane Transport and Metabolism Symposium* (in the Press).

CURRAN, P. F. & SOLOMON, A. K. (1957). *J. gen. Physiol.* **41**, 143–168.

NEWEY, H., PARSONS, B. J. & SMYTH, D. H. (1959). *J. Physiol.* **148**, 83–92.

## Does spironolactone block the renal effects of cortisol?

By J. N. MILLS, S. THOMAS and K. S. WILLIAMSON. *From the Department of Physiology, University of Manchester*

The part played by the adrenal cortex in a variety of homoeostatic and other adjustments can conveniently be studied by the administration of an agent which blocks its effects upon the kidney. Spironolactone (aldactone, Searle, 3-(3-keto-7α-acetylthio-17β-hydroxy-4-androsten-17γ-yl)-propionic acid-γ-lactone) has been shown to block the renal effects of aldosterone, but observations on its antagonism to cortisol are scanty (Kagawa, 1960).

We have collected the urines produced between 10.30 and 12.30 by eleven students, with or without administration of cortisol and/or spironolactone. Cortisol, 100 mg, as hemisuccinate, was injected intravenously at 9.30; spironolactone was given by mouth, 200 mg at bedtime and 100 mg on waking.

Cortisol produced the familiar fall in sodium and rise in potassium excretion. If a subject injected with cortisol had been given spironolactone, sodium excretion was higher but potassium excretion indistinguishable; we infer that spironolactone exerts some blocking action upon the sodium-retaining effect of cortisol, but none upon its kaliuretic effect. If a subject who had taken spironolactone was injected with cortisol, his sodium excretion was reduced; the blocking action is therefore incomplete.

We therefore performed a second experiment on each subject, with double the dose of spironolactone, 300 mg at bedtime, repeated on waking; the results were indistinguishable from those with the smaller dose.

Neither cortisol nor spironolactone consistently altered urine flow or creatine output.

This dissociation between the action of cortisol upon sodium and potassium could be important in understanding the mode of action of this steroid.

REFERENCE

KAGAWA, C. M. (1960). *Endocrinology*, **67**, 125–132.

## Some observations on receptors of the soleus muscle innervated by Group III afferent fibres

By P. Bessou and Y. Laporte. *Laboratoire de Physiologie de la Faculté de Médecine de Toulouse, France*

Most muscle receptors innervated by Group III afferent fibres (myelinated fibres of 4–1 $\mu$ diameter, Lloyd & Chang, 1948), in the cat, are activated by pressure. The pressure receptors are of several kinds which differ by the type of mechanical stimulus to which they respond, by their threshold, rate of adaptation and location (Bessou & Laporte, 1960; Paintal, 1960). More information about some of these receptors has been obtained by exploring the pressure sensibility of exposed soleus muscles in order to delimit the receptive field of single Group III fibres. Measurements of intramuscular conduction times, and adaptation curves of some receptors, were made whenever possible.

Single Group III fibres prepared in dorsal-root filaments were identified by their speed of conduction (less than 24 m/sec) using the conversion factor 6 of Hursh (1939). Tensions developed by stretch or by contraction of the muscle were recorded with a strain-gauge.

Receptors with a small field of activation, of about 3–10 $mm^2$, are found on the surface of the muscle or in the connective tissue around the tendon. They adapt quickly and need strong kneading to be activated. The average speed of intramuscular conduction of their axons is 1–2 m/sec. Stretch or contraction of the muscle does not activate them.

Receptors mostly activated by deep pressure on the junction zone between muscle and tendon have a slow rate of adaptation. They often give a low-frequency discharge during the onset of stretch.

The receptive field of some Group III fibres is surprisingly large. Those fibres are activated by pressure exerted within an area of several square centimetres on the belly of the muscle.

This extensive activation is due, at least partly, to the presence of receptor structures in different parts of the muscle annexed to the same afferent fibre, since partial removal of the fascia or section of the muscle suppresses the activation of that fibre only from the denervated regions of the muscle.

A few spindles and tendon organs innervated by Group III fibres were also identified.

REFERENCES

Bessou, P. & Laporte, Y. (1960). *J. Physiol., Paris*, **52**, 19–20.
Bessou, P. & Laporte, Y. (1960). *C.R. Soc. Biol., Paris* (in the Press).
Hursh, J. (1939). *Amer. J. Physiol.* **127**, 131–139.
Lloyd, D. & Chang, J. (1948). *J. Neurophysiol.* **11**, 199–207.
Paintal, A. (1960). *J. Physiol.* **152**, 250–270.

## Cation exchanges of human red cells

By PADDY D. MCCONAGHEY and M. MAIZELS. *University College Hospital, London, W.C. 1*

Human red cells incubated in solutions of non-electrolytes like lactose, become highly permeable and rapidly lose anion, cation and water. Cation so lost may be replaced by other monovalent cations, and these may be 'sealed' in the cells with $CaCl_2$, which added to a suspension (to 2·5 mM) restores normal cell permeability (for details see Bolingbroke & Maizels, 1959). Such cells, however, lack energizing substrates and will only transport cations against concentration gradients if adenosine is added to the suspension (to 0·04%, w/v), as well as glucose. It thus becomes possible to prepare 'Na cells', 'Na + Rb cells', 'Li + K cells', etc., and to study their cation exchanges on transference to $NaCl + NH_4Cl$, RbCl or similar media (Table 1).

TABLE 1. Cation transfer of red cells treated with lactose solution and resuscitated

| Expt. | Loading medium | Cation concentration of incubation medium (m-equiv/l.) | Cell composition (m-equiv/l. water?) after rocking in the incubation medium: At 0 hr | At 24 hr |
|---|---|---|---|---|
| 1 | Na + K | Na 110, K 20 | Na 60, K 66 | Na 18, K 100 |
| 2 | Na + Rb | Na 110, Rb 20 | Na 58, Rb 68 | Na 23, Rb 97 |
| 3 | Na + Cs | Na 110, Cs 20 | Na 62, Cs — | Na 31, Cs — |
| 4 | Na | Na 110, $NH_4$ 20 | Na 100, $NH_4$ — | Na 46, $NH_4$ — |
| 5*a* | Na + Li | Na 110, Li 20 | Na 67, Li 64 | Na 47, Li 85 |
| 5*b* | Li + Na | Na 20, Li 110 | Na 63, Li 68 | Na 7, Li 126 |

*Note:* Cations are removed from red cells with lactose solution and then replaced by transferring the cells to an electrolyte medium containing suitable salts (loading medium). Cation is sealed in the cells with $CaCl_2$ and the cells transferred to a new 'Incubation medium'.

If pure 'Na cells' prepared in this way are incubated in NaCl medium, it may be shown by means of tracers that influx and efflux are roughly equal at about 5 m-equiv $Na^+$/l. cells.hr. Addition of $K^+$ to the medium evokes movements of $Na^+$ and $K^+$ against the concentration gradients (Expt. 1). $Rb^+$, $Cs^+$ and $NH_4^+$ can replace $K^+$ (Expts. 2, 3 and 4): this confirms the work of Solomon (1952), Post & Jolly (1957) and others, who, however, used cells containing high residues of natural $Na^+$ and $K^+$. In the case of $Na + NH_4$ systems, passive diffusion is very rapid, and no accumulation of $NH_4^+$ can occur to offset active loss of cell $Na^+$. Thus in Expt. 4 $Na^+$ lost was 76 m-equiv/l. cells, and cell volume decreased from 92 to 73% of normal. $Na^+$ concentration was only 46 m-equiv/l. cell water, the osmotic pressure being maintained by the high osmotic pressure of the haemoglobin in concentrated solution. $Li^+$ and $Na^+$ in Li + Na systems move against the concentration gradients, but the movements are small

(Table 1, 5*a*, 5*b*). In Na-free systems (e.g. Li+K; Rb+Cs) no active transport occurs.

REFERENCES

BOLINGBROKE, V. & MAIZELS, M. (1959). *J. Physiol.* **149**, 563–565.
POST, R. L. & JOLLY, P. C. (1957). *Biochim. biophys. acta*, **25**, 118–128.
SOLOMON, A. K. (1952). *J. gen. Physiol.* **36**, 57–110.

## Pyrogen-induced vasodilation in the transplanted kidney

By K. E. COOPER, W. I. CRANSTON, W. J. DEMPSTER and R. F. MOTTRAM.
*M.R.C. Body Temperature Research Unit, Department of the Regius Professor of Medicine, The Radcliffe Infirmary, Oxford, and Department of Surgery, Post-Graduate Medical School, Hammersmith Hospital, London, W.*12

During fever, induced by bacterial pyrogens, there is renal vasodilatation; this is not a direct effect of the pyrogen, and initial attempts to detect a circulating vasodilator agent have failed (Cranston, Vial & Wheeler, 1959). It has been reported that sympathectomy does not affect this renal vasodilatation in man (Goldring, Chasis, Ranges & Smith, 1941) and in dogs (Hiatt, 1942). Because of the difficulty of ensuring a complete

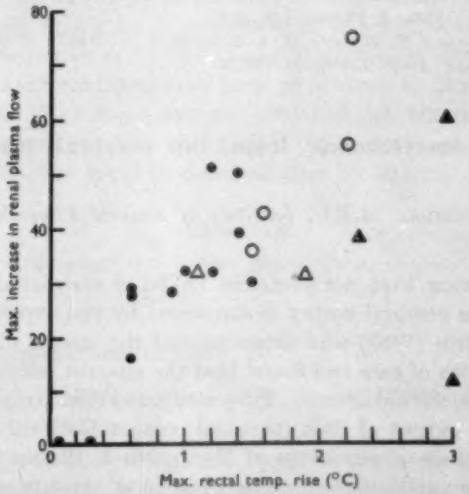



Fig. 1. Maximum increase in renal plasma flow as a percentage of the control value, plotted against maximal rectal temperature rise. Open and filled circles, results after intrathecal and intravenous injections of pyrogen into normal dogs. Filled and open triangles, results after intrathecal and intravenous injections into dog with transplanted kidney.

sympathectomy, observations have been made on a dog which had one kidney auto-transplanted to the pelvis and a contralateral nephrectomy (Dempster, 1954). 'Effective' renal plasma flow was measured by P.A.H. clearance and all measurements were made with the animal unanaesthetized, except for a brief period of anaesthesia (thiopentone 15 mg/kg) to permit cisternal puncture to be carried out. The pyrogen used was a lipopolysaccharide obtained from *S. abortus equi* ('Pyrexal', Wander) in doses of 0·05–0·08 μg/kg. Intravenous or intrathecal injection of this pyrogen caused fever and an increase in 'effective' renal plasma flow; the increase was similar to that observed in normal dogs in the same circumstances (Fig. 1).

When the body temperature of the dog was similarly raised by environmental heating, the 'effective' renal plasma flow was reduced, though the haematocrit rose. Renal blood flow, calculated from these values, was reduced. The arterial pressure was unaffected. It is clear that the renal vasodilatation was independent of the integrity of the nerves to the kidney, and that it could not be attributed to the elevation of temperature alone: a further search for a humoral agent is indicated.

REFERENCES

Cranston, W. I., Vial, Salvador U. & Wheeler, Henry O. (1959). *Clin. Sci.* **18**, 579.
Dempster, W. J. (1954). *J. Physiol.* **124**, 15*P*.
Goldring, W., Chasis, H., Ranges, H. A. & Smith, H. W. (1941). *J. clin. Invest.* **20**, 637.
Hiatt, E. P. (1942). *Amer. J. Physiol.* **136**, 38.

## Release of acetylcholine from the cerebral cortex and the cerebellum

By J. F. Mitchell. *A.R.C. Institute of Animal Physiology, Babraham, Cambridge*

The suggestion that acetylcholine (ACh) is associated with nervous activity in the cerebral cortex is supported by the experiments of MacIntosh & Oborin (1953) who demonstrated the release of ACh from the eserinized cortex of cats and found that the amount released was directly related to the electrical activity. They also found that atropine (1–5 mg/kg) increased the output of ACh (personal communication). In the present experiments these observations of MacIntosh & Oborin have been confirmed on sheep and cats and extended in an attempt to establish the site of ACh release and the factors influencing its release.

Perspex cups enclosing 0·8–3·0 $cm^2$ of the exposed cortex were filled with 0·5–1·0 ml. of Ringer–Locke solution containing eserine ($10^{-4}$ g/ml.) and the solution left in contact with the brain for 10–30 min. The samples were

assayed for ACh, with suitable controls, on the frog rectus or cat blood-pressure preparations. Throughout the experiments an integrated form of the electrical activity of the cortex was used to monitor anaesthesia and to assess the total activity during the collection of samples.

During medium cyclopropane or ether anaesthesia the ACh release from the cortex of cats and sheep, after atropine, was $0{\cdot}11–1{\cdot}0 \times 10^{-9}$ g/min/cm$^2$ cortex and during light anaesthesia the output rose to $1{\cdot}5–3{\cdot}5 \times 10^{-9}$ g. The release was approximately doubled during convulsive activity induced by leptazol (150 mg/kg) reaching $4{\cdot}8–5{\cdot}0 \times 10^{-9}$ g in some animals.

The release from both sides of the cortex was similar and localized electrical stimulation of one side increased the output on both sides two to four times; this increase was affected by changes in the stimulus frequency. Stimulation of the white matter below the collection area also raised the ACh output. The cortical origin of the released ACh is suggested by experiments in which samples were collected after removing the cortex and after local cooling of the cortex; both procedures greatly reduced or abolished the release.

Small amounts of ACh, 5–20 % of those collected simultaneously from the cerebrum, have been detected in samples from the surface of cerebellum; the release from this region, like that from the cerebrum, is increased after leptazol.

The possibility that a large proportion of the ACh released from the cortex was carried away in the blood was investigated in eserinized animals by sampling blood from the sagittal sinus, or in sheep by direct cannulation of cortical veins. The blood samples obtained did not contain ACh in detectable amounts ($> 1{\cdot}0 \times 10^{-8}$ g/ml.). After death, the ACh release fell rapidly until none could be detected after 25–70 min.

REFERENCE

MacIntosh, F. C. & Oborin, P. E. (1953). *Abstr. XIX int. physiol. Congr.* 580–581.

## The foetal haemoglobins of mice

By J. Barrowman and K. B. Roberts. *Department of Physiology, University of Edinburgh*

In mammals, the change from the foetal to the adult type of haemoglobin usually coincides with birth, although small quantities of adult haemoglobin may be present in uterine life and small quantities of foetal haemoglobin may persist for a period after birth.

The haemoglobins of foetal, new-born and adult mice have been studied by electrophoresis, using the starch gel techniques described by Smithies

and modified by Poulik (1957). The haemoglobins are examined in the cyanmethaemoglobin form and the gel stained with *o*-dianisidine. In certain strains of mice, such as C57.Bl.6J., a single haemoglobin may be found in the adult blood, while in other strains, such as CBA, three distinct haemoglobins separate out.

The haemoglobin of the late foetal or the new-born animal of the C57.Bl.6J. strain appears identical to that of the adult, but if foetal blood is taken at 15 days of uterine life then an extra slow-moving component may be seen in the gel. At 12 days a third haemoglobin is present and at this stage the characteristic haemoglobin of the adult is considerably diminished in amount. The replacement of a foetal by the adult type of haemoglobin therefore occurs, in this strain of mice, not at birth but at about 2 weeks of intra-uterine life.

A similar change is seen in CBA mice. In the early foetus (before 15 days), the two fast-moving haemoglobins are present in very reduced amounts, whilst the third, slow-moving component is present in increased amounts. After the 16th day, the pattern becomes indistinguishable from that of the adult. It is again seen that the change from the foetal to the adult pattern does not coincide with birth but takes place two-thirds of the way through pregnancy. This may be associated with the transition from circulating nucleated to non-nucleated erythrocytes which occurs at about the same stage of development (Attfield, 1951).

Some of this work was carried out by one of us (J. B.) in the Roscoe B. Jackson Laboratory, Bar Harbour, Maine, while in receipt of a National Science Foundation grant.

REFERENCES

Attfield, M. (1951). *J. Genet.* **50**, 250.
Poulik, M. D. (1957). *Nature, Lond.*, **180**, 1477.

## Absorption and secretion of electrolytes in the stomach of the rabbit foetus

By G. H. Wright. *Department of Physiology, St Mary's Hospital Medical School, London, W.2*

The experiments were carried out *in vitro* on stomachs from 19 to 31 days gestation age. A glass cannula was tied into the oesophageal stump and the duodenal stump was tied off. The stomachs were filled with the experimental solutions through the cannula. The stomachs were then immersed in bicarbonate-Ringer at 35° C and pH 7·4. The experiments lasted from 3–6 hr.

Net changes in volume of gastric contents were measured as well as changes in amount of Na, Cl and H, which was measured as titratable

acid. The electrical potential difference (p.d.) between the gastric contents and the bathing solution was also measured.

When the stomachs were filled with Ringer's solution there was found to be a p.d. across the gastric epithelium of 15–30 mV (lumen negative). This p.d. was unaltered in sign and magnitude if the internal Ringer's solution was replaced by 150 mM-NaCl. When the internal Na was replaced by choline the p.d. fell in a manner dependent upon gestation age. Thus up to 23 days the p.d. fell to zero when choline replaced Na. However, after 23 days the p.d. only fell to about 30% of its initial value. These effects were quite reversible.

When the internal solution was 150 mM-NaCl there was a decrease in volume of gastric contents and amount of Na and Cl. This occurred at all ages studied. When choline replaced Na there was no volume change observed in stomachs up to 23 days, although a decrease in amount of Cl took place due to passage of this ion down its electrochemical potential gradient. From 23 days onwards there was an increase in volume of contents and amount of H and Cl when choline replaced Na inside. With 150 mM-NaCl inside there was still an increase in amount of titratable acid although Na, Cl and volume of contents decreased.

It was found that all net transfers of Na out of the stomachs occurred against the gradient of electrochemical potential for this ion. Similarly, all net transfers of Cl into the stomachs under conditions of no Na inside took place against the electrochemical potential gradient.

When net water transfer is plotted against net solute transfer a straight line of slope 332 m-osmole/kg water passing through zero is obtained for stomachs of all ages studied.

Thus from 19 to 23 days the foetal stomach is solely absorptive in function, the primary process being active outward transfer of Na at a rate of about 0·8 $\mu$ equiv/hr at 23 days and 18 $\mu$ equiv/hr at 29 days. After 23 days HCl secretion is superimposed on the absorptive process, active inward Cl transfer being involved at a rate of about 1·6 $\mu$ equiv/hr (at 29 days), All water transfer is accounted for by solute transfer.

Menzies (1958) has shown that the only cell type present up to 23 days is an undifferentiated columnar cell, the oxyntic cells appearing on the 23rd day onwards. Thus active Na absorption must be associated with the former cell type and HCl secretion with the latter.

This work was assisted by a grant from the Central Research Fund, University of London.

REFERENCE

MENZIES, G. (1958). *Quart. J. micr. Sci.* **99**, 485–496.

## Viability of mouse bone marrow frozen to −79° C in the presence of dimethyl sulphoxide

By M. J. Ashwood-Smith. *Division of Experimental Biology, National Institute for Medical Research, Mill Hill, London, N.W.7*

Mouse, rabbit and human bone marrow has been preserved at −79° C in presence of glycerol and subsequently administered to irradiated animals (Ferrebee, Billen, Urso, Lu, Thomas & Congdon, 1957; Lochte, Ferrebee & Thomas, 1959). Viability of the thawed specimens was assessed *in vitro* by measuring DNA synthesis. The ability of preserved marrow to synthesize DNA was correlated with survival of erythroid cells. Glycerol-treated mouse marrow showed a 90% reduction in DNA synthesis after freezing.

Bovine erythrocytes are not permeable to glycerol and are not protected by it from haemolysis during freezing and thawing. They are permeable to dimethyl sulphoxide which protects them from the damaging effects of freezing to −79° C. Human erythrocytes and bull spermatozoa are similarly protected (Lovelock & Bishop, 1959). Since then no further studies on protection by dimethyl sulphoxide of living cells at subzero temperatures have been reported.

The toxicity of dimethyl sulphoxide administered intraperitoneally to mice proved low, almost half that of glycerol (Ashwood-Smith, 1960). Mouse bone marrow has therefore been treated with dimethyl sulphoxide and stored at −79° C for future administration to irradiated mice. Viability of the frozen cells has been studied *in vitro* by morphological and biochemical methods.

The marrow was obtained from femurs of mice of the 'TO' strain and suspended in Parker '199' tissue culture medium containing 30% horse serum and varying concentrations of dimethyl sulphoxide up to 15% (v/v). Samples containing about $10 \times 10^6$ nucleated cells in 1 ml. were sealed in ampoules and cooled slowly to −79° C. After 1 hr at this temperature the samples were thawed rapidly at 37° C and examined by phase contrast and ultra-violet fluorescence microscopy on slides coated with agar containing Earle's solution and serum. Under these conditions cells spread out and became actively motile within 30 min at 37° C. Acridine orange at a concentration of 1:40,000 was used for the fluoresence studies of the living cells. Marrow frozen in the absence of dimethyl sulphoxide appeared grossly abnormal. Nuclei were pyknotic in many cells and others had lost all their cytoplasm. The more mature cells of the myeloid series were particularly susceptible to damage. By contrast, a high proportion of cells frozen in the presence of dimethyl sulphoxide in concentrations up to 15% were morphologically normal and moved actively.

Synthesis of protein and lipid depended upon the presence of intact, living cells. Marrow which had been homogenized, or frozen in the absence of dimethyl sulphoxide, showed no incorporation of $^{14}C$ into either the lipid or protein fractions when incubated in Parker '199' containing (1-$^{14}C$) sodium acetate. Marrow cells frozen in Parker '199' containing dimethyl sulphoxide gave incorporation rates up to 50% of control values depending on the concentration of dimethyl sulphoxide.

REFERENCES

Ashwood-Smith, M. J. (1960). *Int. J. Rad. Biol.* (in the Press).

Ferrebee, J. W., Billen, B., Urso, I. M., Lu, W. C., Thomas, E. D. & Congdon, C. C. (1957). *Blood*, **12**, 1096–1100.

Lochte, H. L., Ferrebee, J. W. & Thomas, E. D. (1959). *J. Lab. clin. Med.* **53**, 117–126.

Lovelock, J. E. & Bishop, M. W. H. (1959). *Nature, Lond.*, **183**, 1394.

## The action of local anaesthetics on the action potential of frog sartorius muscle fibres

By M. H. Draper and K. Karzel. *Department of Physiology, University of Edinburgh*

Frog sartorius muscles left in Ringer's solution (114 mM-Na) maintain their resting potential for at least 6 hr at 16° C. Cocaine, procaine and xylocaine produce a slow reversible depolarization of about 5 mV per hr with 5 mM concentrations (Draper, Friebel & Karzel, 1959; Friebel, Draper & Karzel, 1960). In the present studies the mean resting potential of twenty fibres in the companion muscle was measured after each series of action-potential determinations. This precaution eliminated uncertainties due to the possibility of the development of large tip potentials during the experiment (Adrian, 1956).

In view of our experience with the constancy of the membrane potential in resting muscle we were surprised to observe that a far wider variation in resting potential was found in stimulated muscles. The possibility of damage to the membrane by impalement during contraction was tested by measuring the mean resting potential before and after a period of stimulation at 1/sec. It was found that 10 min of such stimulation reduced the mean resting potential by 5–10 mV and that recovery was a matter of hours. If the number of stimuli to any fibre was kept to two or three it was possible to sample ten to fifteen action potentials in a muscle with no demonstrable change in mean resting potential. With these precautions the mean magnitude observed was $130 \pm 0{\cdot}8$ mV ($\pm$S.E. $n = 28$) with a mean overshoot of $42 \pm 0{\cdot}7$ mV. Muscles left for 4 hr in Ringer's solution had a significantly reduced overshoot.

No essential difference in action was found between cocaine, procaine

and xylocaine. The time elapsing from the addition of the drug to the first observation was about 5 min. By this time, with 1 mM drug, the rate of rise of the action potential was greatly slowed, the overshoot was less than 10 mV and the duration, measured at 80% repolarization, was increased from 2 to 6 msec. These changes slowly progressed until the muscle became inexcitable, usually within 1 hr. Just before this, action potentials of 30 mV or less with a duration of 10 msec were observed. The resting potential was usually greater than 80 mV at this time. These changes are similar to those described for a much lower dosage (0·05 mM) in Purkinje fibres, except that the duration of the Purkinje fibre action potential was shortened (Weidmann, 1955).

REFERENCES

ADRIAN, R. H. (1956). *J. Physiol.* **133**, 631–658.
DRAPER, M. H., FRIEBEL, H. & KARZEL, K. (1959). *J. Physiol.* **148**, 61–62*P*.
FRIEBEL, H., DRAPER, M. H. & KARZEL, K. (1960). *Arch. exp. Path. Pharmak.* **238**, 79–80.
WEIDMANN, S. (1955). *J. Physiol.* **129**, 568–582.

## Identification of efferent neurones in the reticular formation of the medulla

By J. H. WOLSTENCROFT. *The School of Medicine, Leeds* 2

## Comparison of bradykinin with other vasodilators in man

By R. H. FOX, R. GOLDSMITH, D. J. KIDD and G. P. LEWIS. *Division of Human Physiology and Division of Physiology and Pharmacology, National Institute for Medical Research, London, N.W.* 3

## Cardiovascular response to exercise in the intact dog and its modification by atropine

By M. J. ALLWOOD, E. W. O. KECK, R. J. MARSHALL and J. T. SHEPHERD. *Mayo Clinic and Mayo Foundation, Rochester, Minn., U.S.A.*

*Continued from front cover*


PAGE

*Draper, M. H. and Karsel, K.* The action of local anaesthetics on the action potential of frog sartorius muscle fibres . . . . . 27P

*Wolstencroft, J. H.* Identification of efferent neurones in the reticular formation of the medulla . . . . . 28P

*Fox, R. H., Goldsmith, R., Kidd, D. J. and Lewis, G. P.* Comparison of bradykinin with other vasodilators in man . . . . . 28P

*Allwood, M. J., Keck, E. W. O., Marshall, R. J. and Shepherd, J. T.* Cardiovascular response to exercise in the intact dog and its modification by atropine . . 28P

and xylocaine. The time elapsing from the addition of the drug to the first observation was about 5 min. By this time, with 1 mM drug, the rate of rise of the action potential was greatly slowed, the overshoot was less than 10 mV and the duration, measured at 80% repolarization, was increased from 2 to 6 msec. These changes slowly progressed until the muscle became inexcitable, usually within 1 hr. Just before this, action potentials of 30 mV or less with a duration of 10 msec were observed. The resting potential was usually greater than 80 mV at this time. These changes are similar to those described for a much lower dosage (0·05 mM) in Purkinje fibres, except that the duration of the Purkinje fibre action potential was shortened (Weidmann, 1955).

REFERENCES

ADRIAN, R. H. (1956). *J. Physiol.* **133**, 631–658.
DRAPER, M. H., FRIEBEL, H. & KARZEL, K. (1959). *J. Physiol.* **148**, 61–62*P*.
FRIEBEL, H., DRAPER, M. H. & KARZEL, K. (1960). *Arch. exp. Path. Pharmak.* **238**, 79–80.
WEIDMANN, S. (1955). *J. Physiol.* **129**, 568–582.

## Identification of efferent neurones in the reticular formation of the medulla

By J. H. WOLSTENCROFT. *The School of Medicine, Leeds* 2

## Comparison of bradykinin with other vasodilators in man

By R. H. FOX, R. GOLDSMITH, D. J. KIDD and G. P. LEWIS. *Division of Human Physiology and Division of Physiology and Pharmacology, National Institute for Medical Research, London, N.W.* 3

## Cardiovascular response to exercise in the intact dog and its modification by atropine

By M. J. ALLWOOD, E. W. O. KECK, R. J. MARSHALL and J. T. SHEPHERD. *Mayo Clinic and Mayo Foundation, Rochester, Minn., U.S.A.*

*Continued from front cover*

PAGE

*Draper, M. H. and Karzel, K.* The action of local anaesthetics on the action potential of frog sartorius muscle fibres . . . . . 27*P*

*Wolstencroft, J. H.* Identification of efferent neurones in the reticular formation of the medulla . . . . . 28*P*

*Fox, R. H., Goldsmith, R., Kidd, D. J. and Lewis, G. P.* Comparison of bradykinin with other vasodilators in man . . . . . 28*P*

*Allwood, M. J., Keele, E. W. O., Marshall, R. J. and Shepherd, J. T.* Cardiovascular response to exercise in the intact dog and its modification by atropine . . 28*P*

THE JOURNAL OF PHYSIOLOGY, JANUARY 1961

VOL. 155, No. 1

## CONTENTS

PAGE

MARLEY, E. AND PATON, W. D. M. THE OUTPUT OF SYMPATHETIC AMINES FROM THE CAT'S ADRENAL GLAND IN RESPONSE TO SPLANCHNIC NERVE ACTIVITY . . . . . 1

McCONAGHEY, PADDY DEBORAH AND MAIZELS, M. THE OSMOTIC COEFFICIENTS OF HAEMOGLOBIN IN RED CELLS UNDER VARYING CONDITIONS 28

TAKEUCHI, A. AND TAKEUCHI, N. CHANGES IN POTASSIUM CONCENTRATION AROUND MOTOR NERVE TERMINALS, PRODUCED BY CURRENT FLOW, AND THEIR EFFECTS ON NEUROMUSCULAR TRANSMISSION. . . . . 46

ANDREW, B. L. THE EFFECT OF CERTAIN ANAESTHETICS ON THE ACTIVITY OF SMALL MOTOR FIBRES SERVING THE HIND LIMB OF THE RAT . . . . 59

FINDLAY, J. D. AND INGRAM, D. L. BRAIN TEMPERATURE AS A FACTOR IN THE CONTROL OF THERMAL POLYPNOEA IN THE OX (*BOS TAURUS*) . . 72

BRAVO, MARIA C. AND FERNANDEZ DE MOLINA, A. FROG'S SPINAL CORD POTENTIALS GENERATED BY STIMULATION OF CUTANEOUS NERVES . 86

HELLER, H. AND PICKERING, B. T. NEUROHYPOPHYSIAL HORMONES OF NON-MAMMALIAN VERTEBRATES . . . . . 98

BURNSTOCK, G. AND HOLMAN, MOLLIE E. THE TRANSMISSION OF EXCITATION FROM AUTONOMIC NERVE TO SMOOTH MUSCLE . . . . 115

SINGLETON, A. G. THE ELECTROMAGNETIC MEASUREMENT OF THE FLOW OF DIGESTA THROUGH THE DUODENUM OF THE GOAT AND THE SHEEP . . 134

JABBUR, S. J. AND TOWE, A. L. ANALYSIS OF THE ANTIDROMIC CORTICAL RESPONSE FOLLOWING STIMULATION AT THE MEDULLARY PYRAMIDS . 148

LLOYD, SYBIL AND PICKFORD, MARY. THE ACTION OF POSTERIOR PITUITARY HORMONES AND OESTROGENS ON THE VASCULAR SYSTEM OF THE RAT 161

HUNT, C. C. ON THE NATURE OF VIBRATION RECEPTORS IN THE HIND LIMB OF THE CAT . . . . . 175

WOLEDGE, R. C. THE THERMOELASTIC EFFECT OF CHANGE OF TENSION IN ACTIVE MUSCLE . . . . . 187

(Contents of *Proceedings* are inside front and back covers)

*Printed in Great Britain at the University Press, Cambridge*
(*Brooke Crutchley, University Printer*)